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Colour Reactions of Flowers attributed to (*a*) Flavanols and (*b*) Carotenoid Oxides

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SUMMARY

1. The petals of many white flowers, when heated with 2N hydrochloric acid, give golden-brown to red-brown colorations, attributable to catechins (yellow-brown) and leuco-anthocyanins (rose to crimson). Extracts of petals which give this reaction give a red colour with vanillin in concentrated HCl, which is consistent with the reaction being due to flavanols.

2. With the exception of two species of Malvaceae, no flowers of herbaceous plants have given this reaction. A majority of flowers of woody plants give the reaction. The limited distribution of flavanols (supposing this identification of the chromogens to be correct) is contrasted with the universal distribution of anthocyanins.

3. The petals of many yellow flowers, when allowed to stand in contact with 1 per cent. methanolic HCl, produce a green coloration attributable to the presence of carotenoid oxides in the petals. Flowers not giving this reaction are limited in their systematic distribution.

THE parts of plants used for food, or otherwise of economic importance, are usually complicated in chemistry and structure. Cultivated plants are also, for the most part, complicated in genetic constitution. For a general study of plant pigments, and the influence of physiological and genetic factors upon them, simplification can be achieved by the study of flowers, especially since these possess the advantage of providing by mere inspection a considerable amount of information with regard to their pigment constitution.

Chromatography now provides an opportunity of making much more complete studies of the constitution of plant extracts, and a start has been made on a chromatographic survey of phenolic pigments and related substances in flowers. In the course of this study several colour reactions have been encountered which seem to have some interest from the point of view both of the nature of the substances responsible for them, and of their systematic distribution. Two of these reactions form the subject-matter of the present communication.

(*a*) A COLOUR REACTION OF WHITE FLOWERS

In preparing extracts of petals for paper chromatography, 1 per cent. methanolic HCl is proving to be a useful general extractant. After macerating the petals in this solvent, the solution is decanted off from the residue and

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applied to the filter-paper as the 'unhydrolysed' extract. A portion of this extract is taken down almost to dryness, a small volume of water is added, and an equal volume of concentrated HCl, to make a solution approximately 5N with regard to HCl (subsequently referred to as 'strong' HCl). This is heated in a boiling water-bath for 5 minutes, in which time glycosides are almost completely hydrolysed. The aglycones are extracted with ethyl acetate, the ethyl acetate solution concentrated, and applied to the paper as the 'hydrolysed' extract. The colour-changes during these operations are noted, and supplement the main reaction with which we are concerned. This consists in suspending either intact petals or the residue from the first extraction in 2N aqueous HCl, and heating in a boiling water-bath. *A 'positive' reaction to the test consists in the petals and the aqueous medium in which they are suspended becoming golden-brown to red-brown after 1–15 minutes' heating.*

As a detailed example the behaviour of *Camellia japonica* may be cited. The methanolic HCl extract of the pure white variety examined, originally pale yellow, became deep orange when concentrated in the water-bath. When strong HCl was added to this concentrate and the solution was heated in the water-bath, a deep chocolate precipitate was formed.

Taken in conjunction with the deep orange-brown discoloration of the petals when bruised, these reactions strongly suggested the presence of catechin tannins, and in fact, when *d*-catechin was subjected to similar treatments, similar (though not identical) results were obtained.

Test	*Camellia japonica* petals	*d*-Catechin
Boiled dil. aq. HCl	Petals and solution became rich orange-brown	Slowly became golden-yellow, with ppt.
This solution extracted with amyl alcohol	Extract golden, becoming crimson on concentration	Extract yellow-brown
Amyl alcohol extract shaken with aq. NH_3	Transiently greenish in aqueous layer, then golden-yellow, remaining yellow in amyl layer on reacidification	Deep red-brown in aqueous layer, becoming yellow-brown again on acidification

The presence of catechin and *epi*catechin in the petal extract was confirmed by two-way paper chromatography. Markers of *d*-catechin and *l*-*epi*catechin were applied at the free edges of the paper on both runs. The catechins were developed with ammoniacal $AgNO_3$ (Bradfield and Bate-Smith, 1950). The results were unequivocal, as shown by R_F values below:

Solvent	Catechin	*Epi*catechin	Unknowns
Phenol	0·42	0·42	0·41, 0·42
Butanol-acetic acid	0·67	0·785	0·645, 0·77

The *epi*catechin component was much the heavier of the two. No other substance was visible on the chromatogram in visible light, in ultraviolet light when treated with ammonia vapour, or when sprayed with ammoniacal silver nitrate, so that it is unlikely that other phenolic substances in substantial quantity were present.

The somewhat redder colour of the reaction with dilute HCl in the case of he petals than in the case of catechin suggests the possibility that in addition o catechins leuco-anthocyanins may have been present in the former. These would be converted into anthocyanidins under the conditions of the reaction and (as will be seen later) can be readily detected, when present, by the usual ests. The transient greenish coloration produced when the amyl alcohol extract was shaken with alkali further suggests the presence of anthocyanidin n the extract. When, however, the pigment was expelled from amyl alcohol nto water by addition of benzene, at least 12 vols. of the latter were needed for complete expulsion, whereas the most required for any anthocyanidin would be 10–11 vols. The main component of the pigment extracted with amyl alcohol was not, therefore, anthocyanidin, and the amount of leuco-anthocyanin in the fresh petal, if any, must have been small in comparison with that of the catechins.

A second variety of *Camellia japonica*, in which the petals responded to bruising less intensely and with a greyish-brown discoloration, proved to contain gallocatechin and *epi*gallocatechin (R_F in butanol-acetic acid 0·585 and 0·46, respectively, compared with 0·57 and 0·47, Bradfield and Bate-Smith, 1950). A considerable trail of, presumably, condensed catecho-tannins was also present on the chromatogram in this instance. Gallocatechin when heated with 2N.HCl gives a grey-brown precipitate dissolving in amyl alcohol to give an olive-brown solution. The discoloration of the petals on bruising is evidently related therefore to the nature of the phenolic precursor substances present.

As an example of red-brown reaction, the behaviour of a white form of *Erica carnea* may be described. The corollas, detached from the other parts of the flowers, rapidly became apricot, then bright red-brown, when heated with 2N.HCl. The colourless precursor in this instance was scarcely extractable from aqueous solution with ethyl acetate, as would have been the case if it had been a catechin. Its behaviour suggested that it was a leuco-anthocyanin, but the final colour was much browner than that of any anthocyanidin.

No species yet examined has, in fact, given a coloured product consisting solely of anthocyanidin; a brown component is invariably present, usually in considerable excess over the red or crimson component. The sequence of colour-change is usually via yellow, golden-brown, and apricot, but, less frequently, the first colour observed may be a clear rose-pink, deepening to crimson-brown.

That leuco-anthocyanins are at least in part responsible for the reaction has been demonstrated in a number of ways.

1. Solutions of the coloured products in amyl alcohol are invariably crimson, whether from golden-brown or red-brown reactions. When shaken with aqueous alkalis a transient green coloration is observed.

2. Expulsion tests with benzene from amyl alcohol (cf. p. 3) have given proportions consistent with anthocyanidins in the following cases:

		Colour of 'reaction'
Abutilon hybridum 'Boule de Neige'	4 vols.	Deep apricot-brown
Dombeya spectabilis	5–6 vols.	Red-brown
Erica carnea	5–6 vols.	Brown-crimson
Raphiolepis indica	4 vols.	Mahogany
,, *ovata*	6 vols.	Brown-pink

3. Chromatography using butanol-2N.HCl as solvent (Bate-Smith and Westall, 1950) has given faint indications of cyanidin in a few instances. The most satisfactory extracts for chromatography were prepared by heating the petals in 5 per cent. ethanolic HCl at the boiling-point of the solvent, and evaporating the extract until the colour was deep enough to apply to the paper. The spots so formed have always been brownish-pink, contrasting strongly with the clear pink of the cyanidin marker. The brown component, which considerably exceeds anthocyanidin in quantity, either flows on the solvent front or remains on the origin. Species in which an anthocyanidin (agreeing with cyanidin simultaneously run) has been visible on the chromatogram are *Dombeya spectabilis*, *Raphiolepis ovata*, and *Erica carnea*; those in which *no* anthocyanidin could be demonstrated were *Campomanesia thea* and *Hardenbergia violacea* (*monophylla*).

The brown impurity does not arise from decomposition of the anthocyanidin once this has been formed, since cyanidin is entirely unaffected by prolonged heating with ethanolic HCl at the boiling-point of ethanol. It is conceivably due to a secondary reaction of the leuco-anthocyanin, similar to the condensation reaction which the catechins undergo, or perhaps also to co-condensation of the leuco-anthocyanin with catechins when these are present.

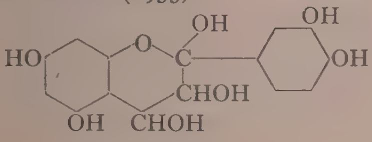
4. A further test which is positive with all species which give the golden-brown to red-brown reaction when heated with acid is consistent with the identification of the precursors as flavanols. This test, the production of a pink colour with vanillin in concentrated HCl, is given by substances containing a phloroglucinol residue, provided that they do not contain a carbonyl group next to the ring. Catechins give the reaction, and can be detected on chromatograms by spraying with vanillin+HCl, but flavones, flavonols, and flavanones do not react. Besides numerous extracts which have been tested by spotting on filter-paper and spraying with this reagent, chromatograms of extracts of the following species, run in butanol-acetic acid, have been developed by spraying with the reagent.

Dombeya spectabilis. Spots developed at R_F 0·57, 0·32.

Erica carnea. Strong reaction at origin, with faint trail up to $R_F \sim$ 0·1.

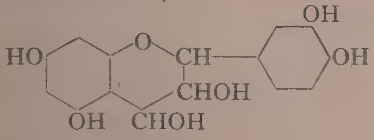
Camellia japonica (2nd variety). Reactions at R_F 0·59, 0·45 corresponding with gallocatechin and *epi*gallocatechin, with trail from origin to $R_F \sim$ 0·3.

There is no certain way at present of distinguishing between the reactions due to unidentified catechins and those attributable to 'leuco-anthocyanins'. The latter are at present definable only as substances which, when heated with 2N aq. HCl or 5 per cent. ethanolic HCl produce anthocyanidins, and they clearly exist in many different forms (cf. Blank, 1947); some, perhaps corresponding with the chromogens present in *Dombeya* and *Raphiolepis*, are uncombined with sugars or organic acids and are readily extracted from aqueous solution with ethyl acetate; others, such as those in *Erica carnea* and *Abutilon* 'Boule de Neige', are glycosidically combined or otherwise complex, and almost insoluble in ethyl acetate. Both the tendency to condense to insoluble, brown-coloured compounds and ability to form anthocyanidins when heated with hydrochloric acid are consistent with a flavanol structure such as that (I) suggested by the Robinsons (1933):



(I)

or (II) (with concomitant oxidation):



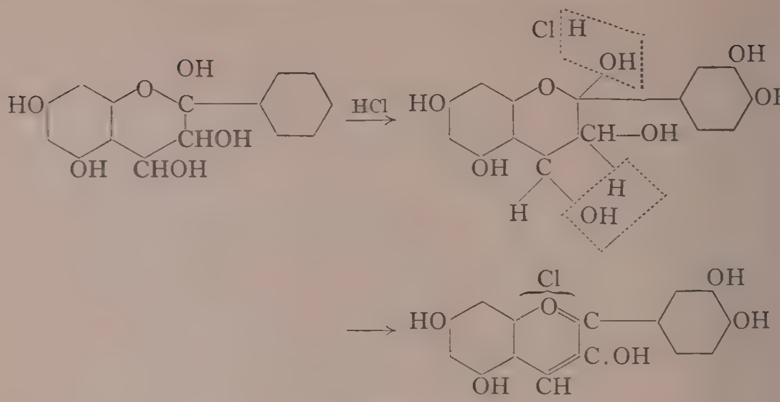
(II)

This last, in diastereoisomeric forms, might well possess the R_F values recorded for the *Dombeya* chromogens.

The structure I is that assumed for the leuco-anthocyanins of *Arachis* seeds by Tayeau and Masquelier (1949*a*), and the brown insoluble component remaining on the chromatogram would correspond with the latter's 'tannoide' (1949*b*).

It should be noted that while catechin possesses two diastereoisomeric forms, both I and II could each exist in four such forms or, including optical isomers, eight isomeric forms altogether. In the case of the catechins, only *d*-catechin and *l-epi*catechin appear to occur naturally. Substances of the formulae I and II might, by analogy, occur in several but not all of the possible isomeric forms, and this could account, in part, for the differences between species in the rate of development, and the shade, of the colour produced. Other differences might be, and probably are, due to glycosidation. Forsyth (1952) reports several different leuco-anthocyanins in cacao beans, some of which contain sugar residues. The expected range of variation is therefore sufficient to account for all the facts observed on the assumption that *the reaction under consideration is due to the presence of flavanols containing one to three hydroxyl groups in the central ring.*

Conversion into anthocyanidin only requires removal of water in the case of structure I ('flavantriol') but requires oxidation in addition to removal of water in the case of structure II ('flavandiol').



Cyanidin chloride

There are numerous instances in which the action of dilute acid in the cold is sufficient to produce anthocyanin pigmentation in white (or acyanic) flowers. Such cases are *Rosa gallica* (Willstätter and Nolan, 1915), *Helenium autumnale* (Hill, 1951), and, observed in the course of the present tests, white forms of *Lupinus polyphyllus*, *Wistaria sinensis*, *Dombeya dregeana*, certain uncommon varieties of *Dahlia variabilis*, and *Primula obconica*. The latter is especially interesting because it was shown that a neutral methanolic extract of the petals does not give a positive reaction with vanillin-HCl. This would indicate that the precursors are structurally quite different in the two cases. In all probability the colourless precursors of anthocyanins developed in the cold are anthocyanins in the 'pseudobase' form, requiring no step of oxidation or dehydration to convert them into the normal coloured compound.

The results of applying the test to a considerable number of white flowers are recorded below:

Woody Species. Positive: *Acokanthera venenata* (v. faint); *Aesculus hippocastanum; Arbutus unedo; Camellia japonica; Campomanesia Thea; Catalpa speciosa; Crataegus oxyacantha; Pyracantha coccinea; Weigela japonica; Dombeya cayeuxii; Dombeya dregeana; Dombeya spectabilis; Erica carnea; Erica melanthera; Erica urceolaris; Hardenbergia monophylla; Hydrangea sargentiana; Ligustrum vulgare; Magnolia denudata; Magnolia stellata; Magnolia virginiana; Mespilus germanica; Olearia haastii* (faint); *Philadelphus coronarius* (faint); *Prunus subhirtella; Sorbus aucuparia; Raphiolepis indica; Raphiolepis ovata; Rhododendron obtusum japonicum* 'Tama-fuyo'; *Ribes laurifolium; Rosa rugosa; Sparmannia africana; Spiraea betulifolia; Wistaria sinensis.*

Negative: *Carpenteria californica; Choisya ternata; Clematis montana* (? weakly positive); *Clematis recta; Colletia cruciata; Datura suaveolens; Deutzia szetchuanensis; Jasminum officinale; Magnolia soulangeana*

'Lennei' (? weakly positive); *Mitriostigma axillare; Nerium oleander* (? weakly positive); *Paeonia* sp. (Tree Paeony); *Passiflora coerulea; Pittosporum tobira; Robinia pseudoacacia; Rosa damascena; Rosa* 'Mermaid'; *Rosa multiflora; Rosa soulieana; Rubus odoratus; Syringa vulgaris; Viburnum opulus; Viburnum rhytidophyllum.*

HERBACEOUS SPECIES. Positive: *Abutilon hybridum* 'Boule de Neige'; *Althaea rosea.*

Negative: *Achillea millefolium; Anemone coronaria; Anemone palmata; Antirrhinum majus; Aquilegia hybrida; Arctotis grandis* × *Venidium fastuosum; Begonia rex* var.; *Bellis perennis; Calceolaria alba; Calystegia sepium; Cerastium tomentosum; Chrysanthemum major; Cosmos bipinnatus; Dahlia variabilis* (vars.); *Datura stramonium; Digitalis purpurea; Fragaria* (cult. strawberry); *Galega officinalis; Helleborus* (garden origin); *Iberis semperflorens; Lamium album; Lupinus polyphyllus; Nicotiana suaveolens; Nigella damascena; Oxalis cathara; Paeonia lactiflora; Papaver nudicaule; Pelargonium zonale* 'Snow Queen'; *Petunia violacea; Phlox decussata; Potentilla rupestris; Primula obconica; Primula sinensis; Senecio cruentus* (Garden Cineraria); *Solanum tuberosum; Symphytum officinale.*

The above arrangement is intended to bring out a feature noticeable at an arly stage of this work, namely that the reaction is virtually confined to the owers of woody species. However, not all woody species give the reaction.

If, as the present evidence seems to indicate, the reaction is due to catechins nd leuco-anthocyanins, it is surprising, considering the universal distribution f anthocyanins in flowers of both woody and non-woody species, that the euco-anthocyanins should be so limited in their distribution. This indicates a istinct separation in the pathways of anthocyanin and leuco-anthocyanin nd catechin) formation, in spite of their apparently close chemical relationhip. The leuco-anthocyanins clearly deserve much closer attention from the lant physiologist than they have so far received.

(*b*) A COLOUR REACTION OF YELLOW FLOWERS

When preparing extracts of flowers for chromatography, as described on . 1, using 1 per cent. methanolic HCl as extractant, it was observed that e extracts of certain yellow or cream-coloured flowers became distinctly reen after standing for an hour or two with the extractant in an open testbe. On further investigation it was found to be quite a common property f flowers of this colour, associated with the presence in them of carotenoid igment. The coloration is not produced when 1 per cent. aqueous HCl is sed as extractant. It is produced, in different species, at different rates and ith varying intensity. When a definite green coloration is present, residual ellow carotenoid can be selectively extracted from the methanolic extract ith light petroleum and it then appears that the over-all green colour is due the formation of a bright blue pigment, scarcely soluble in light petroleum.

This pigment is rather unstable, being destroyed by exposure to direct sunlight or by boiling.

The precursor of the blue pigment is readily soluble in light petroleum. A light petroleum extract of petals giving a positive reaction was floated on methanolic HCl, and the petroleum allowed to evaporate. A positive reaction ensued in the methanolic layer. Results of applying the test are given below:

Species giving a positive reaction: *Abutilon hybridum* 'Golden Fleece'; *Alyssum saxatile; Buphthalmum salicifolium; Buphthalmum speciosum; Calendula officinalis; Cassia tomentosa; Cestrum aurantiacum; Cheiranthus cheiri; Helenium autumnale; Helianthus annuus; Inula helenium; Jasminum nudiflorum; Jasminum primulinum; Lathyrus pratensis; Lindenbergia grandiflora; Linum arboreum; Lonicera periclymenum; Lonicera Standishii; Lonicera tragophylla; Lycopersicum esculentum; Mahonia nepalensis; Oenothera tetraphylla; Pavonia spinifex; Reinwardtia tetragona; Rosa foetida; Sonchus arvensis; Taraxacum officinale; Tropaeolum peregrinum; Viola tricolor.*

Species giving a weakly positive reaction: *Hypericum patulum; Mimulus luteus; Potentilla friederichsonii; Tagetes patula* 'nana'.

Species giving a negative reaction: *Calceolaria scabiosifolia; Cucurbita maxima; Cucurbita pepo; Kerria japonica; Limonium sinuatum* (hybr.); *Primula vulgaris; Rosa* 'Mermaid'; *Verbascum blattaria.*

If the reaction were due to carotenoids known to occur in plant tissues, it might be possible to obtain an indication of their identity by comparing the incidence of positive and negative reactions in the above material with the recorded occurrence of particular carotenoids in the same flowers. Reference to Karrer and Jucker (1950) provides data for eight of the species noted above and for four further species of the same genera as others listed. There is, however, no common factor, or combination of factors, recorded as present in those species giving a positive reaction and not recorded as present in those species giving a negative reaction.

It was thought that a probable explanation of the reaction was the similar colour formed by certain carotenoids with strong aqueous HCl (cf. Karrer and Jucker, p. 7). This agrees also in varying in intensity and stability with the particular carotenoid. It is notable that the epoxides and their furanoid transformation products generally give this reaction, and many of these (e.g. violaxanthin, auroxanthin, xanthophyll epoxide, and flavoxanthin) are reported as occurring, some of them widely, in flowers. A number of species giving positive reactions were therefore tested with concentrated aqueous HCl, and all were found to turn green with this reagent. In this case, of course, the colour was confined to the actual substance of the petals; the aqueous phase usually became deep yellow, due to the formation of oxonium salts of flavones also present.

It is perhaps surprising that 1 per cent. methanolic HCl is an effective reagent for eliciting the reaction (in aqueous solution at least 10 per cent.

Cl is required to produce an appreciable coloration). This indicates that the fective concentration of HCl in methanolic solutions may be much higher an in aqueous solutions of the same nominal concentration.

From the systematic point of view, the negative (or very weak) reactions of e Cucurbitaceae (2 spp.), Rosaceae (3 out of 4 spp.), and Scrophulariaceae 3 out of 4 spp.), and the strong and universally positive reactions of the ompositae (9 spp.) are to be noted. In the case of *Calceolaria scabiosifolia* he carotenoid was atypical, remaining in the methanolic layer when shaken ith light petroleum. This behaviour agrees with that of crocin, and it is nteresting to note that crocetin is the only carotenoid recorded for another ember of this family, *Verbascum Thapsus*. *Mimulus longiflorus*, on the other and, is reported to contain β- and γ-carotene, lycopene, cryptoxanthin, eaxanthin, pro-γ-carotene, and prolycopene—none of these, it will be bserved, being of oxide character. *Mimulus luteus* gave only a very weak, ery long-delayed reaction. *Lindenbergia grandiflora*, the fourth member of his family examined, gave a reaction which was entirely bright blue. There re clearly systematic affinities in the incidence of the reaction, and it may well rove useful in studies of the systematic chemistry of plants.

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Inhibition of Germination in *Kochia indica*

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SUMMARY

Seeds of *Kochia indica* Wight germinate rapidly in shallow water, but their germination is retarded on moist filter-paper. The retardation is traced to a surface-active, saponin-like inhibitor, which is readily leached away in water and is adsorbed by charcoal or soil. Excised embryos may also remain dormant on filter-paper, but if rinsed in water quickly become active. Inhibition is favoured by higher temperature (30° C. as against 20° or less), especially in an atmosphere of oxygen, although once active the embryos grow rapidly in such conditions. When the oxygen concentration is reduced to 5 per cent., germination and growth are markedly retarded, but 5 per cent. CO_2 has little or no retarding effect.

INTRODUCTION

Kochia indica Wight is an annual plant which is locally abundant in and around Alexandria on waste ground, by roadsides and railways and along canal banks. In the autumn, when it flowers and sets abundant seed, it is conspicuous as spreading, grey or dust-coloured bushes, up to a metre or even 2 metres high and wide. It is not a native of Egypt and the time and manner of its introduction were still under discussion when this paper was written.

Early in 1951 large numbers of seedlings were observed in apparently inhospitable conditions on dry bare soil. Seeds collected in the autumn were found to germinate readily though not uniformly when first tested in the laboratory. Further attention was given to the conditions of germination and establishment as it had been suggested in local newspapers that the plant might prove a useful desert fodder plant and the seedlings seemed promising material for experiments on tropic reactions.

It soon became apparent, however, that there was much variation in respect of the initiation and rate of germination. Different methods were therefore tried with the object of securing more even germination under standard conditions. Pricking or abrading the dry seed were partially successful, a large proportion of the seeds so treated germinating rapidly. By May, however, there were signs of abnormality and by August the seeds that had remained dry in the laboratory had lost their viability. This was in itself a fact of some interest in relation to the maintenance of the species in the Egyptian climate.

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As soon as the new season's crop of seed became available at the end of ctober, collections were made, continuing through November and Decem-er 1951, and systematic experiments were begun. Two main lines were llowed, aiming respectively at (1) discovering the best conditions for ecuring rapid and uniform germination, whether for tests of viability or for se as experimental material; (2) determining the duration of viability and the onditions that affect this.

The work described in this paper concerns the first of these aims. It revealed omplexities which may be of general interest, as well as throw light on the onditions required for the persistence of the species in a given locality.

EXPERIMENTAL

The method adopted at first was that commonly employed for germination ests with small seeds, of setting them out on wet filter-paper in Petri dishes. ntact seeds were used as controls for comparison with samples variously reated.

As the data accumulated it became apparent that the controls were very ariable, and it was eventually suspected that the degree of wetness of the ilter-paper might be affecting the results. Seeds were therefore tried actually ubmerged in water a few millimetres deep: the result was practically 100 per ent. germination within 24 hours in every case.

This result immediately suggested the question why seeds which would erminate without delay in water might not do so on damp filter-paper for o days or more. As the seeds quickly swelled, the water-supply was obviously dequate; in fact, drops of fluid accumulated on the upper side of the seeds, s if a deliquescent substance were present at the surface. Other factors than vailability of water must therefore be involved.

Since, however, submergence favoured germination, it seemed unlikely hat gaseous exchange was the primary factor: a substance present in the eed, inhibiting germination on filter-paper but leaching out in water, would o far fit the facts better. It had been observed that on filter-paper a brownish tain quickly spread a little way around the seed, evidently coming from the lackish testa.

It was not clear, however, how a substance in the testa would affect the mbryo. Other observations already made indicated further complications. selection of the experimental data will suffice to illustrate this.

Effect of rupture of the covering membranes. Pricking the dry seed increased he percentage of seeds germinating early, though the results were irregular. brasion with fine emery paper was similarly effective, but a proportion of he embryos suffered damage and this procedure was therefore discontinued nd attention concentrated on more controlled perforation. As pricking the ry brittle seed was a rather drastic method, pricking after soaking on damp ilter-paper was tried for comparison. It was not as quickly effective as dry ricking, as shown in the following example; the data for intact seeds are aken for comparison from an experiment started a day earlier:

TABLE I

Germination of seeds collected 31 October 1951: stored air-dry in the laboratory

Treatment	Total number germinating after					
	1	2	3	4	5	6 days
Expt. started 19/11/51						
Pricked dry (50 seeds)	10	17	36	43	44	49[1]
Pricked after 1½ hours' soaking on damp filter-paper (50 seeds)	3	8	11	38	39	47[2]
Expt. started 18/11/51						
Intact (49 seeds)	3	3	19	21	29	30[3]

Although pricking the soaked seed is far from completely effective when done at the beginning, it is commonly effective without delay at a later stage. After 10 days' dormancy on damp filter-paper many of the seeds may be conspicuously inflated: pricking them then is usually followed by active growth of the embryo within 24 hours.

Inflation develops gradually. The testa proper is a blackish, mostly two-celled, relatively inextensible membrane, which breaks and is left variously localized by the expansion of an inner pellicle. This is probably the embryo-sac wall; it is about 5 μ thick, tough and transparent. The inflation of this membrane shows that it is permeable to water and impermeable to one or more osmotically active solutes within, which gradually accumulate. Digestion of the starchy reserves in the endosperm is an obvious explanation of this accumulation. Non-viable seeds late in the season (from July onwards) in which the embryo is dead but starchy reserves are still present also become inflated, but the inflated seed is more globular because the curved embryo no longer exercises pressure. On the other hand, the turgor and growth pressure of a potentially active embryo would be diminished under the influence of a solution capable of inflating the pellicle, so that pricking an inflated seed would release the embryo from a purely osmotic hindrance by allowing the escape and leaching out of the solution, and it would not be necessary to postulate a specific inhibitor.

In the initial stages, on the other hand, before inflation develops, the evidence of osmotic activity is lacking and delayed germination suggests an inhibiting substance. It is necessary to bear in mind, however, the possible influence of the thick continuous pellicle on the exchange of carbon dioxide and oxygen.

In the experiments already quoted the use is illustrated of the release of embryos from the seed as a test of viability. An early preliminary experiment n a small scale revealed that immediately after collection of seed, at least in he early part of the season, released embryos may not become active for ome days.

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[1] The 50th abnormal.

[2] Two others germinated after re-pricking after 8 days' dormancy; the last was still dormant after 10 days, but the embryo became active after release from the seed.

[3] After 9 days 36 had germinated, the other 13 were still dormant after 11 days; of these the embryos removed from 3 became active within 24 hours; of 10 left intact, 1 germinated after 13 days, 5 after 16 days, another after 18 days; 3 were abnormal.

Expt. started 1 November 1951. Eight seeds, collected the previous day, ut on damp filter-paper: 3 with perianth intact, 4 naked, 1 with testa broken o that the cotyledons became free on soaking though the radicle remained losely invested by its pellicle sheath.

By 4/11/51 no germination: 2 of the naked seeds were left intact, embryos issected from the other 6; all were transferred to clean filter-paper. Two mbryos were active by 9 Nov., 3 more by 10 Nov., the sixth by 11 Nov. The 2 intact seeds were dissected on 9 Nov.: the embryos were active 4 nd 5 days later.

In spite of the small numbers in this experiment, the uniformity of behaviour appears to justify the inference that a period of after-ripening was equired: this was completed in 5 to 7 days after freeing the embryos. The seeds left intact 5 days longer still required 4 or 5 days more before the embryos became active.

The early exposure of the cotyledons of 1 seed did not give the embryo any appreciable advantage over others enclosed completely for the first 3 days. Other similar examples have since been observed. This may well mean that the radicle must be freed (cf. Magnus, 1920); the radicle is the first part of a fully freed embryo to become active, showing geotropic curvature a day in advance of the shoot. In seeds that are split by dry pricking, the embryo often straightens while the radicle is still closely sheathed by the pellicle: the hypocotyl may then elongate somewhat and also the whitish root before any gravitational response occurs, and further growth sometimes ceases for a time.

What is probably another example of inherent dormancy is to be seen in the slower germination of seeds stored air-dry for only 6 days as compared with others from the same collection stored for 19 days: both lots were pricked after soaking (Table II):

TABLE II

Numbers of seeds germinated

Days after soaking . . .	1	2	3	4	5	6	8	9	10	11	12
Expt. 6/11/51 (38 seeds) . .	—	1	1	7	10	14	18	23	26	30	36
Expt. 19/11/51 (50 seeds) . .	3	8	11	38	39	47	—	—	—	—	—

In later experiments it is clear that inherent dormancy has disappeared, the embryos are all potentially active, and dormancy is imposed on them by their micro-environment. The operative factors remain to be distinguished, whether inherent in the seed as the immediate environment of the embryo, or external to it, conditions to which the seed as a whole is exposed.

Experiments with released embryos: evidence for a water-soluble inhibitor. Expt. 1/12/51. Seeds were put to soak on damp filter-paper and the embryos dissected out after soaking for 2 hours, 1 day, or 2 days. After dissection they were well rinsed and set out on clean filter-paper: in all cases they became active within 24 hours after release.

In other experiments when embryos were dissected from the seeds *in situ* and left on the same filter-paper without rinsing, usually a little removed from the testa, they sometimes remained dormant. In some cases soaking in water before dissection appeared to favour dormancy. Such results suggested a water-soluble inhibitor which might impregnate the embryo during soaking, but which, as shown by Expt. 1/12/51 above, is readily leached out if the embryos themselves are rinsed in water. The following experiment illustrates the effect of the moisture factor.

Expt. 22/12/51. Seeds were soaked for 5 hours on wet filter-paper and random samples of 9 embryos were set out without rinsing (*a*) on damp filter-paper raised 1 cm. above the bottom of the receptacle, but with a down-turned edge dipping to the bottom; (*b*) on wet filter-paper on the bottom; (*c*) in shallow water 5 mm. deep in another vessel. Within 15 hours, all 9 embryos of (*c*) were active, but only 1 of (*a*) and 3 of (*b*). After a further 10 hours, 2 more each of (*a*) and (*b*) were active, but the remainder were still inactive after another 14½ hours. Half of each lot were then put into water: they were active within 4½ hours. The others showed no change until 2 or 3 days later.

That the water-supply could be a significant factor in such an experiment may be dismissed: even on filter-paper 1 cm. above the level of the water from which its moisture is derived by capillarity, the slow growth of these embryos should not be limited in a moist atmosphere by their rate of absorption of water. The capillary water is, on the other hand, relatively immobile and diffusion of an inhibitor from the embryo would be slow.

The oxygen factor. The results of the following experiment, designed to test the effect of the moisture factor on pricked and intact seeds respectively, directed attention to the oxygen factor.

Expt. 23/12/51. Seeds were distributed in replicates of 15, some intact (*A*), some pricked after soaking on filter-paper (*B*), (1) in shallow water, (2) on single filter-paper, (3) on 4-fold filter-paper. All those in water were germinating by the following day, both intact and pricked. Of the other pricked seeds, 12 of *B*2 and 9 of *B*3 were then germinating and all germinated within 3 days. Of the intact seeds, none had germinated after 2 days; on 26 Dec. 2 of *A*2 were germinating, none of *A*3; on 29 Dec. 7 of *A*2, 2 of *A*3; on 3 Jan., 8 and 5. Thus, after 11 days, 7 on single and 12 on 4-fold filter-paper were still dormant. At this point all the embryos were dissected out: those of *A*2 were left in the fluid that came from the seeds themselves, with the testas, on a glass slide, covered with a small inverted tube with vaselined edge; those of *A*3 were submerged in a small quantity of water at the bottom of a corked tube. All the embryos of the *A*2 group became active without delay, had reached 9½–10½ mm. from hook to tip within 2 days, and had produced root-hairs. Of the other group 2 appeared inactive and none of the rest had grown to more than 5 mm. from cotyledonary node to root tip, though they showed rvature. The tube was then left on its side, so that the embryos were in a film water spreading along the tube: by next day they were growing as well as e *A2* group. Clearly the differentiating factor between the initial treatment the *A2* and *A3* embryos after dissection was access of oxygen, not concentra- on of inhibitor. Experiments were therefore made with these two factors oth in view.

Oxygen versus inhibitor. A preliminary experiment had shown that when 10 eeds were immersed in a corked tube full of tap-water, holding about 10 ml., ith occasional stirring by inversion and rotation, the oxygen available ufficed for the initiation of germination and for active growth for a time t the temperatures then prevailing (about 17–19° C.). The following experi- ent aimed at distinguishing the effects of inhibitor and reduced oxygen upply.

Expt. 4/2/52. Two tubes, each of about 10-ml. capacity, were fitted with ubber stoppers greased with silicone grease to ensure air-tightness. An xtract of seeds was prepared in boiling distilled water. One of the tubes, *A*, was filled with $\frac{1}{3}$ extract, $\frac{2}{3}$ tap-water; the other, *B*, with $\frac{1}{3}$ boiled distilled ater, $\frac{2}{3}$ tap-water. In each tube were immersed 10 seeds which had been oaked for $\frac{1}{2}$ hour, with changes of water. Both tubes were tightly stoppered ith exclusion of air. Five seeds left over from the preliminary washing were mmersed in a tube full of tap-water and closed with an ordinary cork: of hese, 3 were already germinating after 14 hours, all 5 within $1\frac{1}{2}$ days. On the ther hand, in tube *A* there was still no sign of germination; while in *B*, 1 seed ad begun to germinate, the radicle having emerged and responded to ravity, and 4 showed signs of elongation of the radicle. Even in *B* no further hange was observed during the next 36 hours.

As shortage of oxygen was evidently limiting, the volume of liquid in each ube was reduced by about two-thirds, leaving a depth of 1·5 cm., the tubes hen stoppered and shaken vigorously. After 14 hours in the erect position here was still no change in *A* and only a little elongation of the already erminated seedling in *B*; so both tubes were laid on their sides to increase he contact between liquid and air. Within a further 9 hours, 9 of the 10 seeds n *B* were actively germinating, none of those in *A*. After another 20·5 hours ll the *A* seeds were germinating (one, damaged, was discarded); the average ength of the radicle plus hypocotyl was for *A* (9 seedlings) 1·75 mm., for *B* 10 seedlings) 5·07 mm. After this, lot *B* did little more than maintain their nitial lead over lot *A*, both lots continuing to grow actively. It seems clear hat the extract in *A* had a retarding effect on the initial stages of germination, ut did not markedly retard subsequent growth when the oxygen supply was dequate.

This conclusion was borne out by an extension of the experiment: when the olume of liquid in tubes *A* and *B* was reduced, 4 ml. of each discarded liquid as transferred to two small open glass vessels, *AA* and *BB*, reaching a depth f about 7 mm. Ten seeds were submerged in each without previous washing. fter 14 hours the germination record was: *AA*, 1+?2; *B*, 3+?3, with another embryo escaped and so discarded. Within 44 hours all 10 in *AA* were germinating, 8 in *BB*. The odd one in *BB* remained dormant and after 3½ days in all had become inflated: when pricked it quickly became active (cf. pp. 12). The remainder in *BB* maintained their lead over those in *AA*: after 3½ days the 6 largest in *BB* ranged from 10 to 14 mm. (average 12·5 mm.), compared with 8·5 to 12·5 in *AA* (average 9·5 mm.).

Parallel with *A* and *B*, two tubes, *A′* and *B′*, were started a day later (5/2/52), with liquids prepared as before, but closed by corks, and seeds were put in each without previous washing. (Washing might not only have a leaching but a waterlogging effect and remove attached air bubbles.) As there was no sign of germination after 24 hours in either tube, nor 9 hours after admission of a little air, the contents of each tube were transferred to open vessels (about 2·8 cm. in diameter). The subsequent results are given in Table III (times are after transference):

TABLE III

	Number germinating after			Average length (and range) a	
Treatment	4½ hrs.	13½ hrs.	24½ hrs.	24½ hrs.	38½ hrs.
A′ (9 seeds)	—	3+?1	9	4·1 mm. (2–6 mm.)	7·4 mm. (5¼–9½ mm.)
B′ (10 seeds)	3	9	10	7·2 mm. (5–9½ mm.)	13·9 mm. (8–19 mm.)

Here again the extract retarded germination, and growth also seems to have been reduced; but the initial delay before transference is attributable chiefly to shortage of oxygen.

Experiments with continuous aeration. In the next series of experiments a continuous current of air was maintained in order to simplify the conditions from the physical standpoint. The availability of oxygen to the embryo in stationary vessels depends on the initial concentration in the water, volume of water, distance of the air-water surface affecting diffusion, convection currents accelerating access, and rate of solution of gaseous oxygen in the water at the surface of contact with air. The interpretation of results can therefore be complicated. With an air current the air-water surface is multiplied, increasing the rate of entry of oxygen into solution, and the water is kept in motion, reducing the diffusion path consistently. Between parallel replicates therefore the oxygen factor should not differ appreciably. Accessibility of oxygen to the embryo will, however, increase after emergence of the radicle. There may also be competition for oxygen between seeds in the same small volume of water which could act to retard further those starting later.

Seeds were immersed in tubes similar to those used for *Expt. 4/2/52*, two-thirds full of water, and air was bubbled through by way of capillary tubes from reservoirs of known composition. As a rule 15 seeds were put in each tube.

When 5 per cent. of CO_2 was added to the air current, no significant effect was observed when compared with a control with ordinary air, all but 1 or 2 of the 15 seeds germinating in both within 24 hours.

It was otherwise when the oxygen content was reduced to about 5 per cent. by admixture with nitrogen. After 23 hours with a current of ordinary air 13 out of 14 seeds had germinated, with 5 per cent. oxygen only 6 out of 15; the average length of the latter was only 1·5 mm. as compared with 4·7 mm. for the best 6 of the controls.

In such experiments a foam formed above the liquid in the tubes and when stronger extracts of seeds were tried a copious froth emerged from the top of the tube, where it eventually dried to a white scum. This accumulated conspicuously during the first 24 hours. Later the foaming diminished as the constituent responsible for it was progressively removed.

With strong extracts foaming was accompanied by a clouding of the liquid by what appeared to be a flocculent precipitate. When, however, the pressure of the air above the turbid liquid was reduced it effervesced vigorously and became clear again. Similarly, if the dry scum were mixed with water it did not immediately dissolve on shaking, though after long standing it did dissolve; but if the pressure was reduced, vigorous effervescence occurred as with the turbid extract and the substance went into solution.

It appears therefore that the turbidity may be due to the formation of minute air bubbles, each with a 'skin' of the surface-active constituent, and that the scum is similarly composed (unless some more intimate form of 'occlusion' is involved).

With a current of nitrogen, foaming was less striking and turbidity much slower to appear. Whether these phenomena are wholly dependent on access of oxygen is not clear. In an experiment to test this point, tall narrow test-tubes were used, so that the access of atmospheric oxygen was much diminished. With 15 seeds in distilled water and a current of air, turbidity developed within the hour, scum was accumulating in the upper part of the tube within 3 hours, and bubble formation was becoming irregular. In a similar tube, with a current of nitrogen, there was still no sign of turbidity or scum formation and the bubbles were uniform in size and breaking regularly before they reached the top of the tube; by the following morning, however, the liquid was turbid and scum had accumulated.

Two other similar tubes were set up containing extracts made by boiling 15 seeds in distilled water and leaving to cool overnight. Foaming and scum formation were less in these than in the others, both with air and with nitrogen. It appears therefore that boiling diminishes the amount or in some way modifies the constituent responsible for foam formation.

The detergent, foam-producing properties suggested a saponin. Appropriate tests were therefore applied both to extracts and to samples of scum.

Boiling-water extract gave a definite Prussian blue coloration with ferric chloride and potassium ferricyanide. A red coloration also gradually developed after addition of concentrated sulphuric acid. Other tests applied gave negative or uncertain results.

Basic lead acetate gave a copious flocculent precipitate which, after separation of the lead as sulphide, dissolved to form a brownish solution similar to the original extract; this frothed on shaking and gave the Prussian blue test.

The solid scum also gave the Prussian blue test and the red colour with strong sulphuric acid.

Removal of inhibition by adsorption. It appeared probable that a surface active inhibitor, such as these experiments indicate, would be readily adsorbed and that soil itself might have this effect. The following experiment confirms this both for soil and for charcoal and shows clearly that inhibition on filter-paper must be due to local accumulation of a substance which does not diffuse away rapidly enough laterally.

Expt. 17/4/52. Random samples of 15 seeds each were set out on the following substrata in Petri dishes: (1) four layers of filter-paper; (2) garden soil (the basis of this was Nile silt); (3) garden soil thoroughly washed to remove osmotically active substances; (4) filter-paper lying on garden soil; (5) filter-paper lying on washed garden soil; (6) filter-paper on washed charcoal; another sample (7) was submerged in shallow water. Except for (1), all the seeds germinated within 48 hours: differences that occurred within the first 24 hours were of doubtful significance. In marked contrast were the results on filter-paper alone: no seeds had germinated after 48 hours and the last not till after 13 days. The data for number of seeds germinated were as follows: after 3 days, 2; 4 days, 6; 7 days, 12; 8 days, 13; 11 days, 14; 13 days, 15.

Effect of salinity. As *Kochia indica* has been recorded from saline soils in India, it is not surprising that in the last experiment washing the soil had no appreciable effect on germination. An earlier experiment had in fact already shown that even with 1 per cent. NaCl in the soil-water seeds could germinate, though accurate quantitative data were not recorded:

Expt. 13/2/51. Seeds were sown in Petri dishes on well-washed sand, wetted with a range of concentrations of NaCl solution. A high percentage germinated on 0·5 per cent. NaCl, though germination was slower than with water. On 1 per cent. NaCl roughtly 50 per cent. germinated. On the other hand, 2·5 per cent. NaCl entirely prevented germination; but even 5 per cent. NaCl did not injure the seeds, for after 5 days they were still able to germinate when the salt was washed out with water.

Effects of higher temperature and undiluted oxygen. Brief reference must suffice to some early experiments which opened up additional complications which it has not yet been possible to follow further. The results so far described were all obtained at the temperature of the laboratory, usually between 17° and 19° C. or in some of the later ones at 20° C. in an incubator. As the temperature in Alexandria even in spring may rise at times considerably higher and in summer may reach 30–35° C., germination was tried at 30° C. Also, as the possibility was envisaged that oxygen supply might limit the growth of embryos while enclosed in the testa, some samples were put in in atmosphere of oxygen at the same temperature. A summary of the results follows.

Expt. 18/11/51. Lots of about 50 seeds were set out on damp filter-paper, some at the temperature of the laboratory, others at 30° C. in an incubator. Some of the seeds had been collected 18 days, others only 6 days previously. Of the earlier collection 74 per cent., of the later 50 per cent., had germinated after 8 days at the lower temperature. At 30° C. germination was much retarded: after 4 days, none of the later collection had germinated, as against 24 per cent. at the lower temperature; of the earlier collection 8 per cent. as against 45 per cent.

Expt. 26/11/51. Seeds of the later collection were used: they had been collected 14 days previously. All were put in the incubator at 30° C., some in air, others in oxygen.

Of 2 lots of 10 embryos dissected out after 1½ hours' soaking (at room temperature) on moist filter-paper, all those in air were active by the following day, only 4 of those in oxygen. The remaining 6 remained dormant in oxygen for 7 days, but became active within 24 hours after the oxygen was replaced by air.

Two lots of 16 seeds were dissected after 24 hours on moist filter-paper: of those in air 10 germinated within 24 hours, 5 more after 6 days, and the last after 7 days; of those in oxygen only 4 germinated within 24 hours, the remainder were still dormant after 7 days but became active within 24 hours after transference to air.

In all cases those embryos that became active immediately grew just as well in oxygen as in air; and the growth rate was much higher at 30° C. than at the temperature of the laboratory. The effect of the higher temperature on the rest, further accentuated by the effect of 100 per cent. oxygen, was therefore to prevent them from breaking dormancy, or to induce dormancy.

All the intact seeds remained dormant in oxygen at 30° C., and in air only 2 had germinated out of 28 after 7 days.

Pricked seeds. In air, 15 out of 30 germinated within 24 hours, two more by next day; the rest remained dormant for several days longer, but after 7 days all but 3 necrotic seeds had germinated. In oxygen, on the other hand, only 2 germinated within 24 hours, the rest were still dormant after 7 days; but all germinated without delay after transference to air.

Expt. 1/12/51. Some additional data obtained in this experiment but not mentioned earlier (pp. 13–14) are relevant here. In addition to those at room temperature, seeds were soaked at 30° C., some in air, some in oxygen. The embryos after dissection from the testa were thoroughly rinsed in water and set out at 30° C., those from oxygen now in air, those from air, some in air, some in oxygen. All the embryos were active within 24 hours (apart from an odd necrotic one).

It is clear from these results that the effects of higher temperature and of oxygen in retarding germination depend on the presence of a water-soluble substance, readily removed by rinsing in water.

DISCUSSION

The main conclusion that emerges from this work is that moist filter-paper is an unsuitable substratum for germination tests with seeds of *Kochia indica*, owing to local accumulation of a substance which interferes with germination.

Other cases are known of accumulation of inhibitors in filter-paper. It is not to be assumed, however, that poor germination on filter-paper is always to be attributed to this cause. When comparison is made with seeds submerged in water, oxygen concentration may also be a factor.

Morinaga (1926, *a*, *b*) attributed the better germination of white clover seeds in distilled water to the lower oxygen concentration. Similarly, seeds of *Typha latifolia* germinated poorly if at all in air, but well when the oxygen concentration was reduced by admixture with 40 to 80 per cent. of nitrogen or hydrogen. Seeds of *Cynodon dactylon* also germinated better in a reduced oxygen concentration. However, the reverse is more generally true. Morinaga records the failure of many species to germinate submerged in distilled water. In a recent study of five halophytic species of *Atriplex* in Australia, Beadle (1952) notes that for successful germination the seeds must be in contact with air, not even surrounded with a film of water (this is in marked contrast with *Kochia indica*, a member of the same family and also at least tolerant of moderate salinity).

In many species, however, there is good evidence of the presence of inhibiting substances. Borriss (1936) obtained no germination with *Vaccaria pyramidata* seeds on filter-paper in Petri dishes at 20° C., whereas in soil practically 100 per cent. germinated within a few days. Leaching with water removed the inhibition; so also did adsorbents, including charcoal and soil; but the adsorbents acted almost equally well when separated from the seeds and filter-paper by an air gap (cf. Konis, 1940). The inhibitor itself is therefore volatile and it seems possible that it is produced continuously from another substance which is removable by leaching. As boiled extracts of *Kochia indica* seeds retard germination, the inhibitor can in this case hardly be volatile nor be produced by enzymatic action.

Vahl (1940) with *Poterium spinosum* found 2 to 4 days' delay in germination on filter-paper as compared with moist charcoal: here again the inhibitor was volatile. Cox, Munger, and Smith (1945) found evidence of a non-volatile, water- and alcohol-soluble inhibitor in cabbage seed coats. Shuck (1935) also found that certain varieties of lettuce fail to germinate on moist filter-paper at 25° C. in the light, but will do so on moist absorbent cotton or in water. Germination of successive lots of seed without changing the water or washing the cotton results in progressive lowering of the percentage of seeds germinating.

A number of workers have investigated the germination of sugar-beet and found evidence of inhibitors of different kinds. Extracts of 'seed-balls', which reduce the germination of other seeds as well as those of beet (Fröschel, 1939, 1940), were observed by Fröschel to foam strongly, but he did not find he inhibitor markedly adsorbed by charcoal. Duym *et al.* (1947) found evidence of a volatile constituent, accounting for a small part of the inhibition observed, but traced most of it to substances that were dialysable and still active after incineration, that is to inorganic salts. Stout and Tolman (1941) attribute inhibition to ammonia production; while de Kock and Hunter (1950) were able to extract with purified ether an unsaturated yellow oil, water-soluble, with a powerful inhibiting action, e.g. on cress seeds: the inhibition was reversed by washing with water.

Few of the inhibiting substances have been identified chemically, but from the characters described it is clear that they are various (Evenari, 1940; Evenari *et al.*, 1942). Apart from those which may act purely osmotically (including sugars in the juice of succulent fruits, see Oppenheimer, 1922; Ulrich, 1939), little is known of their modes of action, though de Kock and Hunter (1950) find that their ether-soluble inhibitor from sugar-beet affects the oxidative metabolism.

The mode of action of the inhibitor in *Kochia indica* might well prove to be exceptional, as its physical and chemical properties appear to be, and should be worth further investigation. Red beet 'seed-ball' extract does not foam as strongly as extracts of *Kochia indica* seeds, and whether or not sugar-beet is more comparable in this respect there is no evidence that foam production and inhibition are correlated in sugar-beet.

It would seem possible, given a larger supply of seed, to obtain sufficient of the active substance by foam production and subsequent purification to identify it chemically and to obtain further information about its properties, especially its behaviour towards oxygen. This is particularly desirable because of the possibility that the formation of a scum over seeds on filter-paper might effectively reduce the oxygen supply and delay germination in this way. On the other hand, there is already some evidence that the extract reduces the rate of growth, not only of *Kochia indica* seedlings but also of wheat radicles.

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Growth Inhibition Studies

I. MEASUREMENT OF THE EFFECTS OF INHIBITORS ON THE GROWTH RATE OF FLAX SEEDLINGS

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SUMMARY

A method for measuring the rate of growth of small seedlings under special conditions suitable for inhibition and reversal studies is described, and its use is illustrated in studies of the inhibitory action of a variety of chemical substances.

The limitations imposed, in such studies, by the osmotic pressure of the test solutions have been examined in the case of flax seedlings.

INTRODUCTION

THE action of chemical growth inhibitors on the tissues of higher plants has been the subject of much research in recent years. Among the inhibitors studied, those which have been found to act as antimetabolites in their effects on bacteria, moulds, and animals are well represented. Some indication is apparent in this work that these inhibitors act on plants by mechanisms not essentially different from those already suggested for other organisms. Thus, paralleling the work of Woods (1940) with bacteria, there have been reports (Hazard, 1944; Audus and Quastel, 1948; Schopfer and Anker, 1949) that the inhibitory action of sulphanilamide on the sprouting of seeds could be reversed with *p*-aminobenzoic acid; and corresponding to the results obtained by Strandskov and Wyss (1946) with bacteria it has been found that the inhibitory action of thiouracil on sprouting can be reversed with uracil and thymine (Ber, 1949; Trotter, 1949).

However, the analysis of the reversal effects has not, in general, been investigated as fully as in the case of the micro-organisms. One difficulty in the way of such analysis has been the lack of a convenient quantitative method for studying growth rates of plants. Techniques such as that used by Audus and Quastel (1947) or the methods of isolated tissue culture used by Robbins (1922) and White (1934) are very time-consuming, and the method of Thimann (1941) using isolated sections of oat coleoptile does not recommend itself for extensive comparative studies.

The present paper contains a description of a method which has been developed for the purpose of making inhibition and reversal studies with

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seedlings, and describes the results obtained in experiments intended to form a basis for reversal studies which will be reported separately.

Seeds of flax, *Linum usatissimum* L., have been used as the test organism in this investigation.

METHODS

The assumption underlying the general procedure is that when seeds are sprouted in the dark, with water and air as the sole nutrients, the only way they can increase their substance is by the uptake of water. Under these limiting conditions, therefore, water uptake may be regarded as a measure of growth comparable to the increase in wet weight.

In the present experiments this measurement has been made volumetrically using special tubes constructed as indicated in Fig. 1. These consisted of a sprouting chamber (A) to which, by way of a capillary U-tube, was sealed a 1·00-ml. pipette graduated in 0·01-ml. divisions. The distal end of the latter was bent upward, as shown, for a length of about 4 cm.

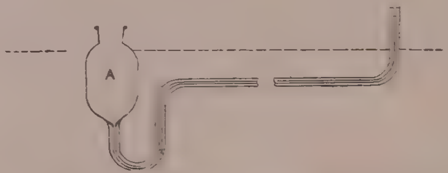

FIG. 1. Tube used for measurement of water uptake.

A small disk of filter-paper (1·5 cm. diameter) was placed in the bottom of A, moistened with a drop of water, and forced down into a conical shape with the end of a stirring rod. A slurry of washed sand, about 20-mesh, in the solution to be tested was then poured in until its level was about 1 cm. higher than that of the graduated side-arm. By gentle tapping the sand was formed into a compact layer, free of air bubbles. More of the solution was added to displace all air from the capillary U-tube and side-arm, the tube being tilted to drain off the excess liquid. Finally, with the graduated tube again horizontal, the meniscus was brought to a convenient position for reading by touching the surface of the sand lightly with a small roll of filter-paper. The sand was thus kept wet by capillarity, which also maintained the position of the meniscus.

To reduce evaporation the upturned end of the pipette was fitted with a short piece of glass capillary (1·0 mm. bore, 2·0 cm. long) held on by gum rubber tubing. A similar piece of capillary tubing inserted in a single-holed rubber stopper was used as a closure for the sprouting chamber. This arrangement served to keep the pressure in A at that of the atmosphere.

A set of such tubes mounted on a brass rack with spring clips was immersed to the level indicated by the dotted line in Fig. 1 in a water-bath at 25·0±0·1° C. Light was excluded from the bath by means of a metal lid.

Condensation inside the lid was conveniently avoided by placing an infra-red amp at such a height above the lid as to maintain its temperature just above that of the bath.

Seeds placed on the surface of the wet sand in the measuring tubes absorbed water, the uptake of which was indicated by the movement of the meniscus in the side-arm. Preliminary tests showed that the water uptake by flax seeds as a function of time followed the pattern of Fig. 2, i.e. there was a rapid initial absorption which diminished in rate until about the 20th hour, when a second period of rapid absorption set in. This second part of the curve was practically linear until about the 60th hour. Direct observation of the seeds under similar conditions indicated that visible sprouts appeared at about 20 to 24 hours. The first part of the curve appeared to represent simple imbibition of water by the dry seed, and was not observably altered by inhibitors at any of the concentrations tested. The effect of an inhibitor (sulphanilamide) was found to be an alteration in the rate of water uptake during the linear phase, 20 to 60 hours. The slope of the water-absorption curve in the interval 30 to 50 hours was selected as a measure of the growth rate for the experiments reported here.

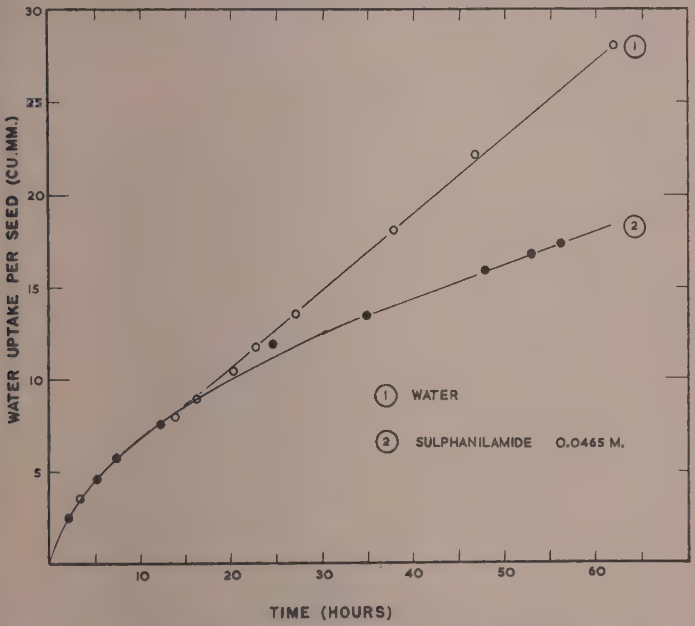

FIG. 2. Water uptake by flax seedlings during sprouting on distilled water (curve 1) and in the presence of 0·0465 M. sulphanilamide (curve 2).

Since the volume changes during the first 20 hours were irrelevant for these experiments there was no necessity for the seeds to be in the measuring tubes during this interval. They were accordingly sprouted in the dark at 25·0°

in Petri dishes containing three layers of filter-paper (Whatman No. 1) and 10 ml. of the test solution. During this interval the measuring-tubes were assembled and allowed to equilibrate in the bath. Besides the considerable saving of time this procedure had the advantage that, since cracked or damaged seeds were more easily detected after soaking for a few hours, it was possible to select 30 sound specimens from each Petri dish, and to ensure virtually 100 per cent. sprouting. At about the 24th hour the seeds were transferred rapidly with the aid of a microscope slide and a small spatula to the measuring-tube containing the same test solution and were distributed evenly over the surface of the sand. The tubes were then restoppered and volume readings made from the 30th hour onward.

Since some evaporation took place through the two capillary openings of the measuring-tubes, a blank correction was necessary. This could be determined during the equilibration period before the seeds were introduced. However, an alternative procedure was adopted. Tests with four different inhibitors showed that at concentrations which completely suppressed sprouting for at least a week there was always a small apparent water uptake which was almost the same in all cases. The average figure, 0·040 mm.^{3} per seed per hour for these experiments, was regarded as a more satisfactory blank correction which included not only the aforementioned correction for evaporation but also any water absorption not associated with sprouting. This constant quantity has therefore been subtracted as a blank in all cases.

For the inhibition curves the slopes of the water-uptake curves, calculated by the method of least squares and corrected for the blank, have been expressed as a percentage of the control rate similarly corrected.

EXPERIMENTAL

Effect of benzimidazole on the rate of growth of flax seedlings. It was considered desirable to make a direct comparison of the results obtained by the method outlined in the foregoing section with those obtained by some independent method. The inhibitory action of benzimidazole was therefore studied both by the water-uptake method and by direct measurement of overall seedling length in samples of 20 seedlings each, withdrawn at intervals from Petri dishes in which the seeds were sprouted in the dark at 25·0°, on layers of filter-paper soaked in the various test concentrations of benzimidazole. The results of the two procedures are given in Figs. 3 and 4.

The slopes of the linear portions of the curves in the interval 30 to 50 hours, calculated by the method of least squares, have been expressed as a percentage of the control rates on water, and these are plotted in Fig. 5 against the logarithm of the benzimidazole concentration. The two methods, it will be noted, yielded similar values for the concentration producing 50 per cent. inhibition of growth rate.

Effect of osmotic pressure on sprouting. In order to carry out studies of reversal of inhibition it was important to know at what concentration to expect the appearance of non-specific inhibition due to the osmotic pressure of the test

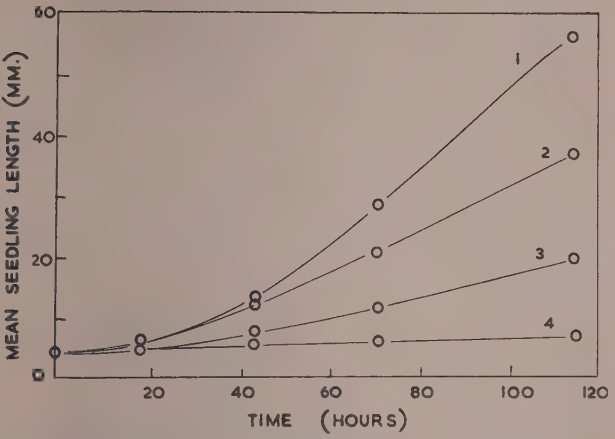

FIG. 3. Effect of benzimidazole of molarities shown on the elongation of flax seedlings: curve 1, o (control); curve 2, $1\cdot06 \times 10^{-3}$; curve 3, $4\cdot24 \times 10^{-3}$; curve 4, $8\cdot48 \times 10^{-3}$.

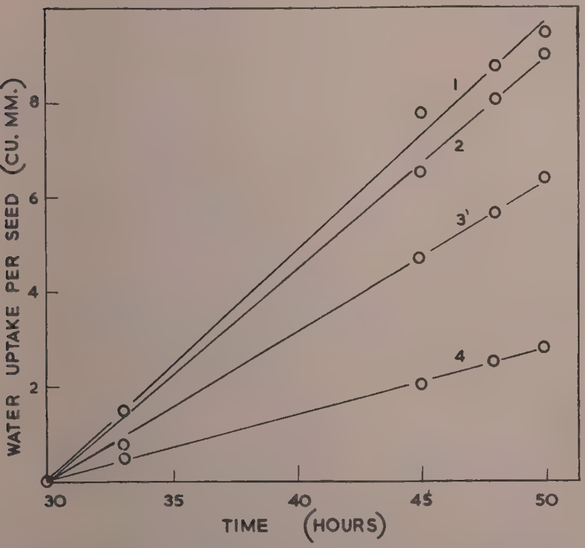

FIG. 4. Effect of benzimidazole of molar concentrations shown on water uptake by flax seedlings: curve 1, o (control); curve 2, $5\cdot29 \times 10^{-4}$; curve 3, $2\cdot12 \times 10^{-3}$; curve 4, $4\cdot24 \times 10^{-3}$.

solution. To investigate this question four compounds were selected for which no specific inhibitory action was anticipated; these were glucose, sucrose, urea, and sodium chloride. The inhibition curves for these compounds are given in Fig. 6, and the concentrations which produced 50 per cent. inhibition of the rate of water uptake are given in Table I.

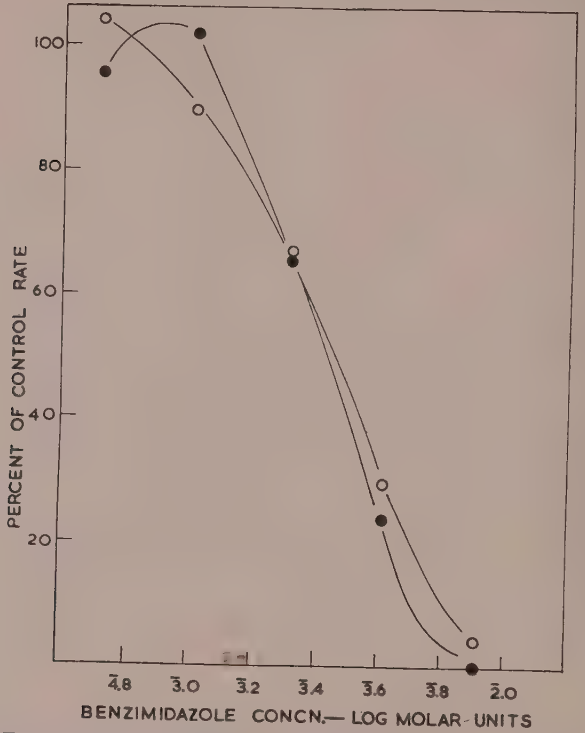

FIG. 5. Inhibitory effect of benzimidazole on the rate of elongation of flax seedlings (open circles) and on their rate of water uptake (solid circles).

TABLE I

Effect of osmotic pressure on sprouting

Compound	Concentration producing 50% inhibition (molal)
Glucose	0·24
Sucrose	0·24
Urea	0·24
Sodium chloride	0·09

The three non-electrolytes differed widely in their molecular weights, yet the molal concentrations causing 50 per cent. inhibition were the same within experimental error. If the inhibition were due to the high osmotic pressure f these solutions it would be expected that a strong uni-univalent electrolyte vould show a comparable effect at about half these concentrations. The bserved value, 0·09 molal, for sodium chloride is regarded as supporting his interpretation, allowance being made for the error of estimate.

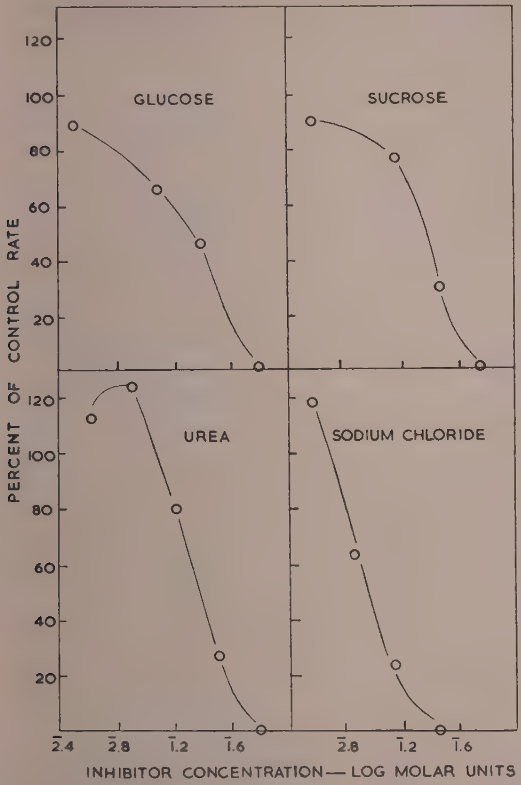

FIG. 6. The inhibitory effects of glucose, sucrose, urea, and sodium chloride on the rate of water uptake during sprouting of flax seeds.

In none of the inhibition studies with flax seedlings has growth been observed in solutions with osmotic pressures in excess of the maximum values tested in this experiment. In the absence of evidence to the contrary it is therefore considered that the inhibitory action of the four compounds listed in Table I was due to osmotic effects and that these impose an upper limit of concentration beyond which reversal studies cannot profitably be pursued. In the case of flax this limit is about 0·1 molal for non-electrolytes.

Effects of various inhibitors. The inhibition curves for a number of different compounds were determined by the water-uptake method. In Table II are summarized the results of these experiments.

TABLE II

Results of inhibition tests

Compound	50% inhibitory concentration (molar)
Chloroacetamide . . .	$3{\cdot}15 \times 10^{-4}$
β-Indolylacetic acid (Na salt) .	$5{\cdot}37 \times 10^{-6}$
Malononitrile	$5{\cdot}13 \times 10^{-4}$
Phenoxyacetic acid (Na salt) .	$4{\cdot}17 \times 10^{-3}$
Sulphanilamide	$1{\cdot}04 \times 10^{-4}$ [1]
2-Thiouracil	$1{\cdot}32 \times 10^{-4}$
dl-Tryptophane	$8{\cdot}91 \times 10^{-4}$
Uracil	$8{\cdot}0 \times 10^{-4}$

[1] This figure is for 30% inhibition. In this case, owing to the presence of a plateau in the inhibition curve, the concentration for 50% inhibition is undefined.

DISCUSSION

The validity of the water-uptake method as a measure of growth rate is dependent on the condition that the seeds are sprouted in the dark on nothing but water. Under these circumstances flax seeds develop visible sprouts at 25° in about 20 hours and the seedling attains an overall length of about 1·5 cm. within the 50-hour experimental period. This growth is eventually limited by the supply of food stored in the cotyledons, but while it is proceeding any net increase in weight must be due to water uptake, since respiration *per se* leads to a net decrease in weight under the experimental conditions. Any water uptake unassociated with growth was eliminated by the blank correction which was obtained for seeds incubated with inhibitor solutions.

The results with glucose, sucrose, urea, and sodium chloride indicate that inhibition due to osmotic effects does not occur at concentrations less than about 0·1 molal for non-electrolytes. Thus osmotic effects may be neglected in the experiments with the other inhibitors reported here.

The fact that the water-uptake method gives results in close agreement with those obtained by one of the more conventional methods is seen in the benzimidazole experiments, in which a direct comparison was made with a method involving measurement of seedling length. It should be noted that the agreement holds for five different concentrations of the inhibitor and that each point on the curves corresponds to a rate measurement extending over a period of 20 hours during which the rate was constant. Such agreement can hardly be regarded as fortuitous.

The procedure described in this paper offers a number of advantages for inhibition studies with seedlings. The measurements can be made on samples of a reasonable size by a method which permits the making of any desired number of readings without disturbing the sample in any way. The time necessary for a complete run is 3 days, in the case of flax, but a considerable number of tubes may be run simultaneously, since each measurement requires only a few seconds. Estimation of the rate of water uptake is simple, since the progress curves are linear throughout the period of measurement.

The wide variation in inhibitory action indicated by the data in Tables I nd II illustrates the sensitivity of the flax seedling to differences in chemical tructure. The strong inhibitory action of β-indolylacetic acid may be noted. Of particular interest are the strong inhibitory effects of tryptophane and uracil, both natural metabolites for some organisms. The results with tryptophane are in accord with the observations of Audus and Quastel (1947) on cress seedlings.

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Growth Inhibition Studies

II. SULPHANILAMIDE INHIBITION AND ITS REVERSAL

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SUMMARY

In a study of the inhibitory effect of sulphanilamide on the growth of flax seedlings and its reversal by *p*-aminobenzoic acid (PABA) and related compounds, two distinct types of reversal mechanism have been observed. The results, derived from growth-rate measurements, are interpreted as indicating that, in the flax seedling, sulphanilamide interferes with the conversion of PABA to pteroyl-glutamic acid.

None of the purine or pyrimidine compounds tested showed any reversing action.

INTRODUCTION

INTENSIVE research during the past decade into the effects of inhibitors on the respiration and growth of organisms has had as one of its important objectives the elucidation of the mode of action of antibacterial and pharmacological agents and of certain toxic compounds. In the course of this work there has been an increasing emphasis on the relation between the effect of an inhibitor on enzymes and its observed effect on the organism. In many cases of inhibition the minute amounts of the inhibitor which are effective, together with the nice structural specificity which they show, has pointed strongly to a mechanism involving enzymes, and in a number of cases work with isolated enzymes appears to have confirmed this hypothesis. As examples one might recall the specific effects of *iso*propylfluorophosphate on cholinesterase (Bodansky, 1946), of malonate on succinodehydrogenase (Quastel and Wooldridge, 1928), and of ephedrine on amine-oxidase (Gaddum and Kwiatkowsky, 1938).

Those instances of inhibition which can be relieved by the simultaneous presence of a second compound have a special interest from the standpoint of biochemical kinetics. Firstly, the fact of reversal implies an essentially intact metabolic machinery and eliminates from consideration an inhibitory mechanism attributable to widespread, non-specific destruction of tissues or of enzymes. Secondly, the mode of action of the inhibitor becomes susceptible of analysis: the specificity of reversal can be examined and quantitative studies made to determine the type of reversal mechanism.

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The present study of the inhibitory action of sulphanilamide on the growth f flax seedlings was undertaken with the intention of analysing its reversal y *p*-aminobenzoic acid (PABA) and related compounds in some detail in rder to compare the reversal picture with that found in the numerous studies hich have been made with bacterial species as test organisms. Hazard (1944) eported that *p*-aminobenzoic acid was capable of reversing the inhibitory ction of sulphanilamide on flax seedlings and others have made similar bservations. However, so far as the author is aware, no quantitative studies ave appeared in which the influence of these compounds on the growth ates of seedlings have been measured directly.

MATERIALS

The sulphanilamide and *p*-aminobenzoic acid used in this investigation ere commercial supplies (Eastman) which had been further recrystallized rom water after a charcoal treatment. The pteroic acid and pteroyl-glutamic cid were kindly supplied by Dr. T. H. Jukes of the Lederle Laboratories.

The *p*-aminobenzoyl-glutamic acid was synthesized by the method of andsteiner and Van der Scheer (1935) from L-glutamic acid. The product, fter charcoal treatment, was recrystallized from water to a constant melting-oint (172·0–172·5° C.) and had a specific rotation $[\alpha]_D^{20} = +28\cdot5^\circ$ in 0·1 N. odium hydroxide.

The adenine and guanine were prepared from yeast ribonucleic acid by the ethod of Jones (1920). They were purified by repeated recrystallization as he sulphate and chloride respectively. Uracil was synthesized by the method f Davidson and Baudisch (1926). The thymine was obtained from Schwartz aboratories, and the adenosine and guanosine from Nutritional Biochemicals Corporation. The cytosine, kindly supplied by Dr. G. C. Butler, was synthe-ized by the method of Hilbert (1930).

Wherever necessary the various test solutions were adjusted to pH 6·8 with ilute sodium hydroxide before use in reversal tests.

METHODS

All determinations of growth rate were made by the procedure described n paper I of this series (Moore, 1952). In brief, the flax seeds were sprouted n the dark at a constant temperature of 25° on sand wet with the test solution nder conditions such that their water uptake could be continuously measured. t has been shown (loc. cit.) that under these conditions the slope of the linear urves of water uptake in the interval between the 30th and 50th hour of prouting can be used as a convenient and reproducible measure of the rowth rate.

Inhibition curves were determined by expressing the calculated slopes of uch lines, after a blank correction, as a percentage of a suitable control, and lotting these percentages against the log. of the sulphanilamide concentration.

EXPERIMENTAL

Inhibitory action of sulphanilamide. The inhibition curve for sulphanilamide was determined as already described. All concentrations were tested i triplicate. The measured water uptakes are given in Table I as examples o typical results. The corresponding inhibition curve is included in Figs. 1 to 4

TABLE I

Inhibitory effect of sulphanilamide on growth-rate of flax seedlings

Tube No.	Sulphanilamide concentration (molar)	Volume change per seed 30 hrs.	34 hrs.	46 hrs.	50 hrs.	Calcd. slopes	Mean slope	Mean slope less blank	% of control rate
	$\times 10^{-3}$		cu. mm.			(cu. mm./seed/hour)			
1	0	0	0·67	5·30	6·40	0·337	0·383	0·343	100·0
2	0	0	0·90	5·60	7·20	0·368			
3	0	0	1·50	6·87	8·87	0·444			
4	0·04	0	1·17	5·83	7·30	0·371	0·349	0·309	90·1
5	0·04	0	1·17	5·60	6·83	0·349			
6	0·04	0	0·83	5·10	6·33	0·329			
7	0·08	0	1·00	5·00	6·10	0·312	0·324	0·284	82·8
8	0·08	0	1·17	5·23	6·50	0·328			
9	0·08	0	1·40	5·47	6·57	0·331			
10	0·16	0	1·03	3·93	4·43	0·227	0·245	0·205	59·7
11	0·16	0	1·20	4·26	5·07	0·254			
12	0·16	0	0·93	4·23	4·97	0·255			
1	0·20	0	0·99	4·10	4·90	0·248	0·243	0·203	52·7
2	0·20	0	1·01	4·07	4·83	0·252			
3	0·20	0	0·92	3·90	4·47	0·229			
4	0·40	0	0·89	3·83	4·30	0·222	0·231	0·191	49·6
5	0·40	0	0·93	3·93	4·57	0·233			
6	0·40	0	0·95	4·07	4·43	0·238			
7	0·80	0	0·94	3·90	4·27	0·222	0·234	0·194	50·4
8	0·80	0	0·99	4·37	4·73	0·247			
9	0·80	0	0·93	4·00	4·50	0·232			
10	1·60	0	0·88	3·90	4·20	0·220	0·231	0·191	49·6
11	1·60	0	0·98	4·30	4·70	0·244			
12	1·60	0	0·91	4·03	4·37	0·228			
13	0	0	1·03	4·87	6·17	(0·258)	0·425	0·385	100·0
14	0	0	1·68	6·53	8·47	0·419			
15	0	0	1·72	7·17	8·50	0·431			

Note: The above data were obtained in two separate runs.

Reversal of sulphanilamide inhibition. In order to test the possible reversin effect of various compounds the inhibition curve was redetermined in th presence of a range of concentrations of the test substances. The effect of *p*-aminobenzoic acid, pteroic acid, pteroyl-glutamic acid, and *p*-aminobenzoyl-glutamic acid are illustrated in Figs. 1 to 4. The following compounds were found to have no detectable reversing action at any test concentration up to that given in parenthesis after the name of the compound:[1] adenine ($4{\cdot}9 \times 10^{-4}$ M.), guanine ($1{\cdot}0 \times 10^{-3}$ M.), uracil ($4{\cdot}5 \times 10^{-3}$ M.), hymine ($4{\cdot}0 \times 10^{-3}$ M.), cytosine ($4{\cdot}5 \times 10^{-3}$ M.), adenosine ($3{\cdot}7 \times 10^{-4}$ M.), and guanosine ($3{\cdot}5 \times 10^{-4}$ M.).

DISCUSSION

The reversal curves obtained in this investigation are clearly of two distinct ypes (cf. Figs. 1 to 4). *p*-Aminobenzoic acid, pteroic acid, and *p*-aminobenzoyl-glutamic acid behaved in the competitive manner; pteroyl-glutamic acid, on the contrary, behaved non-competitively.

If it be assumed that the sulphanilamide is inhibiting some essential enzymatic reaction in the flax seedling, then, employing reasoning similar to that advanced by Shive and Macow (1946) in their theory of 'Inhibition Analysis', one may conclude that the first three compounds act like precursors of the inhibited reaction, and that pteroyl-glutamic acid behaves like a product of the inhibited reaction.

The behaviour of pteroic acid and *p*-aminobenzoyl-glutamic acid, both of which were slightly less effective than *p*-aminobenzoic acid as reversing agents, suggests the possibility that one or both might act by being broken down to *p*-aminobenzoic acid in the organism. The possibility of ascribing the effect of these compounds to *p*-aminobenzoic acid present in them as an impurity can be ruled out in the case of *p*-aminobenzoyl-glutamic acid from melting-point and optical rotation data. In the case of pteroic acid the small amount of material available precluded an assay for *p*-aminobenzoic acid. It may be noted, however, that there would have to be 18 per cent. *p*-aminobenzoic acid present in it to produce the observed effect. In this connexion it is of interest that Lampen and Jones (1946) in some work with pteroic acid found diazotizable amine equivalent to 6 per cent. *p*-aminobenzoic acid in a commercial sample. In any event it is impossible from the present experiments to determine whether either of these compounds is a normal intermediate in the synthesis of pteroyl-glutamic acid by the flax seedling.

The qualitative difference in the reversal curves for pteroyl-glutamic acid as compared with the other reversing agents is most striking. This difference, together with the fact that pteroyl-glutamic acid was more effective than *p*-aminobenzoic acid on a molar basis in reversing the effect of the higher concentrations of sulphanilamide, makes it impossible to attribute the effect of this compound to the presence of *p*-aminobenzoic acid as an impurity or to suppose that it owes its activity to being broken down to the latter in the organism.

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[1] These concentrations were the maximum possible either because of solubility limitations or because the compound itself became inhibitory at higher concentrations.

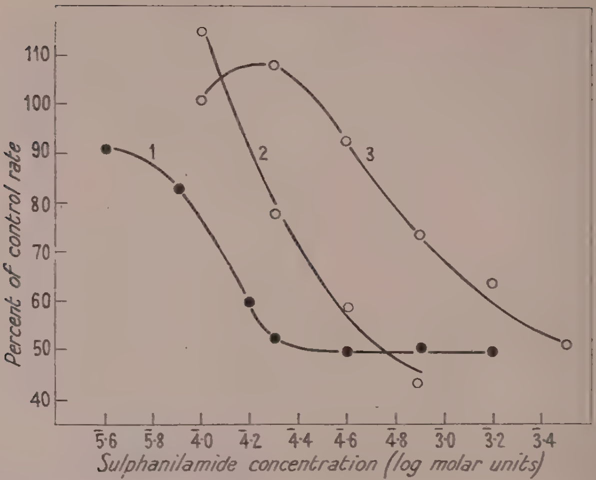

FIG. 1. Reversal of sulphanilamide inhibition with *p*-aminobenzoic acid. Curve 1 is the primary inhibition curve; curves 2 and 3 were obtained in the presence of *p*-aminobenzoic acid, $2{\cdot}92 \times 10^{-5}$ M. and $1{\cdot}46 \times 10^{-4}$ M. respectively.

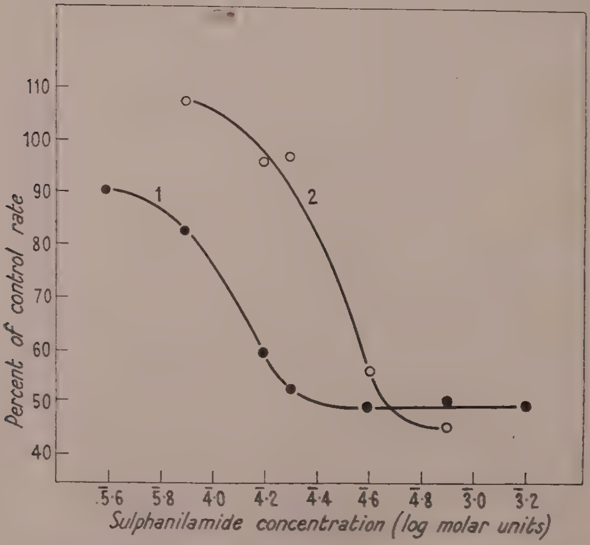

FIG. 2. Reversal of sulphanilamide inhibition with pteroic acid. Curve 1 is the primary inhibition curve; curve 2 was obtained in the presence of $2{\cdot}75 \times 10^{-4}$ M. pteroic acid.

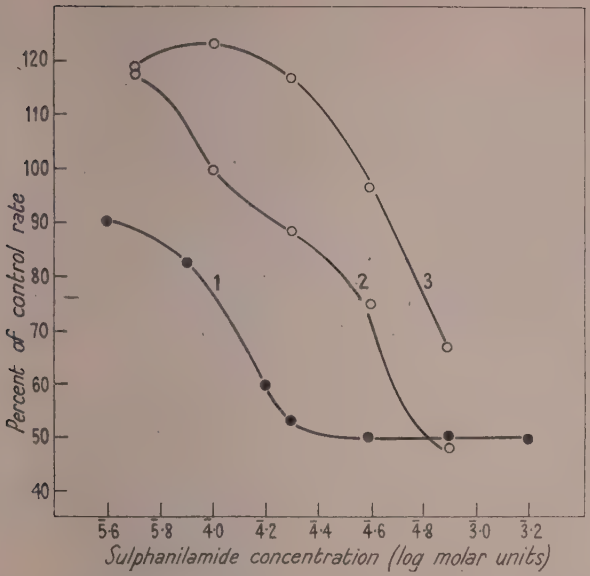

FIG. 3. Reversal of sulphanilamide inhibition with *p*-aminobenzoyl-glutamic acid. Curve 1 is the primary inhibition curve; curves 2 and 3 show the response obtained in the presence of 10^{-4} M. and $2{\cdot}5 \times 10^{-4}$ M. *p*-aminobenzoyl-glutamic acid respectively.

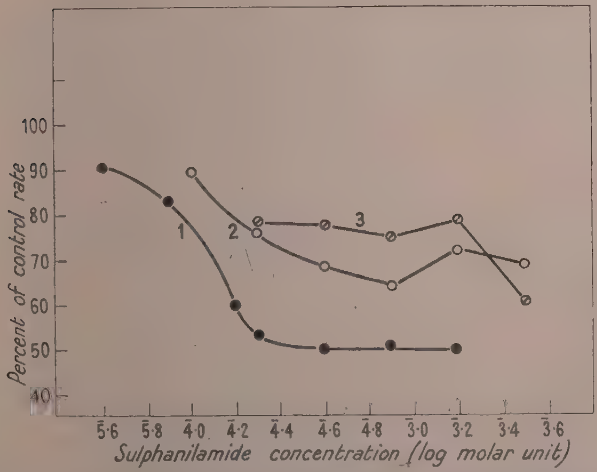

FIG. 4. Reversal of sulphanilamide inhibition with pteroyl-glutamic acid. Curve 1 is the primary inhibition curve; curves 2 and 3 show the response obtained in the presence of $6{\cdot}00 \times 10^{-6}$ M. and $1{\cdot}25 \times 10^{-5}$ M. pteroyl-glutamic acid respectively.

The most probable interpretation of these observations would appear to b that, in the case of flax seedlings, sulphanilamide interferes with the conversion of *p*-aminobenzoic acid (PABA) to pteroyl-glutamic acid (PGA) Whether pteroic acid or *p*-aminobenzoyl-glutamic acid (PABG) act as intermediates in this synthesis cannot be decided from the present data.

$$\text{PABA} \rightarrow \begin{bmatrix} \text{Pteroic acid} \\ \text{PABG} \end{bmatrix} ? \rightarrow \text{PGA}$$

If either pteroic acid or PABG is an intermediate, the fact that they both gave competitive reversal curves would appear to place the point of inhibition between them and PGA rather than between them and PABA.

The complete failure of any of the purine or pyrimidine compounds tested to produce any detectable reversal should also be noted. There have been several reports that thymine or certain other compounds in the group were effective as reversing agents for the sulphanilamide inhibition in the case of certain bacteria. It may well be that species differences are responsible for the different results in the present instance. It may be remarked, however, that the claims for these secondary reversing agents have hitherto been based upon studies of extent of growth at some fixed time rather than upon growth rate. Consequently, as the author has pointed out elsewhere (Moore and Boylen, 1952), they must be considered as of uncertain significance.

The form of the primary inhibition curve for the sulphanilamide is itself reminiscent of the non-competitive reversal curves. It is the picture one might expect if the seeds contained preformed pteroyl-glutamic acid. It is of interest that Woolley and Sprince (1945) found that flax seed meal contains appreciable amounts of a compound of the folic acid type. An alternative explanation would be afforded by the assumption that the reaction inhibited by sulphanilamide, at the concentrations used in these experiments, is just one of two independent pathways to pteroyl-glutamic acid, and that the remaining route has about half the capacity required for the maintenance of the normal growth rate.

ACKNOWLEDGEMENT

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Electron Microscopic Investigations of the Walls of Green Algae

II. THE CYTOPLASM-WALL RELATIONSHIP IN FREEZE-DRIED VALONIA MACROPHYSA

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WITH PLATES I–VI

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SUMMARY

The observations made previously in the electron microscope on formalin-preserved *Valonia* have been substantially confirmed on freeze-dried material. In addition, a further observation has been made which seems to furnish some slight clue concerning the mechanism of cellulose microfibril orientation.

INTRODUCTION

IN a recent article in this journal Preston and Kuyper (1951) presented a preliminary investigation of some of the details of molecular architecture in the walls of *Valonia ventricosa* as seen in the electron microscope. This involved examination of the outermost, intermediate, and innermost lamellae in a number of vesicles. The condition of the first two types of lamella may be taken as established, but the nature of the material available renders the interpretation of the observations on the innermost lamella somewhat uncertain. As pointed out in this first paper, the vesicles had been preserved in formalin for a number of years. It is not to be expected that this would have any major effect on the details of purely cellulosic bodies even as seen in the electron microscope, but the possible effect on the proteins in the cytoplasm and on the protein-cellulose associations at the innermost lamellae is by no means easy to estimate. It was therefore thought essential to make parallel observations on these lamellae in material collected fresh and freeze-dried immediately. It is not, of course, necessarily true that freeze-drying gives any more reliable fixation than does treatment with formalin and the investigation was not projected with any such idea in mind. The aim is rather to compare the observations possible after two very different kinds of fixation.

The present paper describes the observations which have been made on freeze-dried vesicles of *Valonia macrophysa*. Attention is confined solely to the innermost lamella, partly because it is there that the greatest interest lies at the moment and partly because it is not surprising that the outer and intermediate layers presented precisely the same appearance as they did in the

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[1] On leave from Philips Electrical Ltd., Eindhoven, Netherlands during this investigation and now returned there.

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arlier study. It may be well, therefore, to state briefly here the major observa-ions and conclusions which were made on the innermost lamella in formalin-reserved vesicles. These were:

(*a*) The bulk of the cytoplasm appears to have no organized relationship to the cellulose microfibrils of the wall lamella in process of deposition. It usually appears as an amorphous flocculum, pierced with holes or thin places, but is often granular.

(*b*) The underlying microfibrils, composing the lamella in process of deposition, appear to be oriented almost at random.

(*c*) Here and there granular cytoplasmic deposits appear to adhere more firmly to the wall, and these are clearly associated with the microfibrils in that many microfibrils appear to end (or begin) within these deposits.

(*d*) These deposits were therefore identified provisionally as 'islands' of cellulose microfibril synthesis, the mechanism of orientation remaining obscure.

It remains therefore to be determined whether any or all of these can be onfirmed with freeze-dried material.

MATERIAL AND METHODS

Vesicles of *Valonia macrophysa* were collected in the Bay of Naples and reserved, in sea water, still attached to the substrate until further treated ater in the same day at the Stazione Zoologica in Naples. The treatment was s follows. Each vesicle in turn was stripped from its substrate, pierced by the eedle of a hypodermic syringe, and the vacuolar sap removed. By holding he syringe with the needle vertically upwards, the vesicle was then imme-iately inflated with air without removal of the needle. It was then dropped nto a container cooled in a mixture of solid CO_2 and acetone and held there ntil transfer to the freeze drier. After freeze drying the material was stored n tightly stoppered bottles containing calcined calcium chloride and carried ack to England. The period between collecting and observing in the electron nicroscope was in no case longer than 2 weeks. The method of stripping and hadowing was exactly as described in the earlier paper (Preston and Kuyper 951).

RESULTS

The general appearance of the cytoplasm in this material is illustrated in lates I and II. Whenever the cytoplasm is thin enough to be penetrated by lectrons it appears to be composed largely of granules which are of the general rder of twice the diameter of the microfibrils, i.e. about 400–500 Å (Pl. I). therwise the cytoplasm gives the impression of an amorphous mass pierced y holes of variable diameter (Pl. II), although the suggestion of a granular nake-up is always present. This material is therefore not markedly different n this respect from the formalin-preserved specimens used in the earlier nvestigation.

Here and there on any particular preparation areas can be found with various densities of cytoplasmic deposits, and it may be said in general that the areas with thinner deposits present one of two appearances. One type of observation is illustrated in Plate I and again in Plate III*a*. Here the cytoplasm appears literally to be a deposit upon the wall with no visual relation to the microfibrils. This is very evident in Plate I where, however, the magnification is rather low. Even at higher magnifications, although there are, here and there, faint suggestions of a connexion between cytoplasm and wall (Pl. III*a*), the overall appearance is of complete discontinuity between them.

The second type of appearance is illustrated by Plates III*b*, IV, V, and VI. Even at lower magnifications (Pl. III*b*) the cytoplasmic deposits and the cellulose microfibrils appear inextricably mixed; and at higher magnifications even with relatively dense deposits (Pl. IV) fibrillar protrusions from essentially granular masses are very evident. Occasionally areas appear from which the cytoplasm has been removed almost completely (Pl. V). Here the fragments which do remain attached to the underlying cellulose lamella appear to be entangled with cellulose microfibrils. In almost all cases in which this association between wall and cytoplasm is evident, the cellulose microfibrils with which the association takes place are arranged more or less at random.

So far, therefore, these observations resemble very closely those made on the formalin-preserved material. Indeed the only marked difference would seem to be that whereas the so-called ‘islands’ of synthesis were found to be more or less isolated in the earlier observations, they appear to be clustered with the present material so that one could refer to vaguely defined areas in which the cytoplasm is more closely associated with the wall and equally ill-defined areas in which it is less closely associated.

One observation has, however, been made which was not noticed with the formalin-preserved specimens (Pl. VI). This can be made sporadically at the edge of an area in which the association between wall and cytoplasm is fairly close. At such an edge, material which still, from its general features, would appear to be cytoplasmic, tends to aline itself into fibrils, or strings of granules, in a direction almost at right-angles to what are presumably microfibrils in the wall layer below.

CONCLUSIONS

In general, therefore, the present results substantiate the conclusions drawn in the earlier paper and nothing need be added to what was written at that time. Observations of the type illustrated in Plate VI are, however, new and perhaps call for some comment. It would obviously be quite unsafe to attempt any detailed interpretation yet, but the observations made in the earlier paper and the rest of the illustrations presented here leave the mechanism of microfibril orientation so obscure that it may perhaps be permissible to indulge in a little speculation. The general appearance of areas like that illustrated in Plate VI allows the suggestion to be made that across any area of cellulose microfibril production there sweeps a phase of ‘orientation’ following which

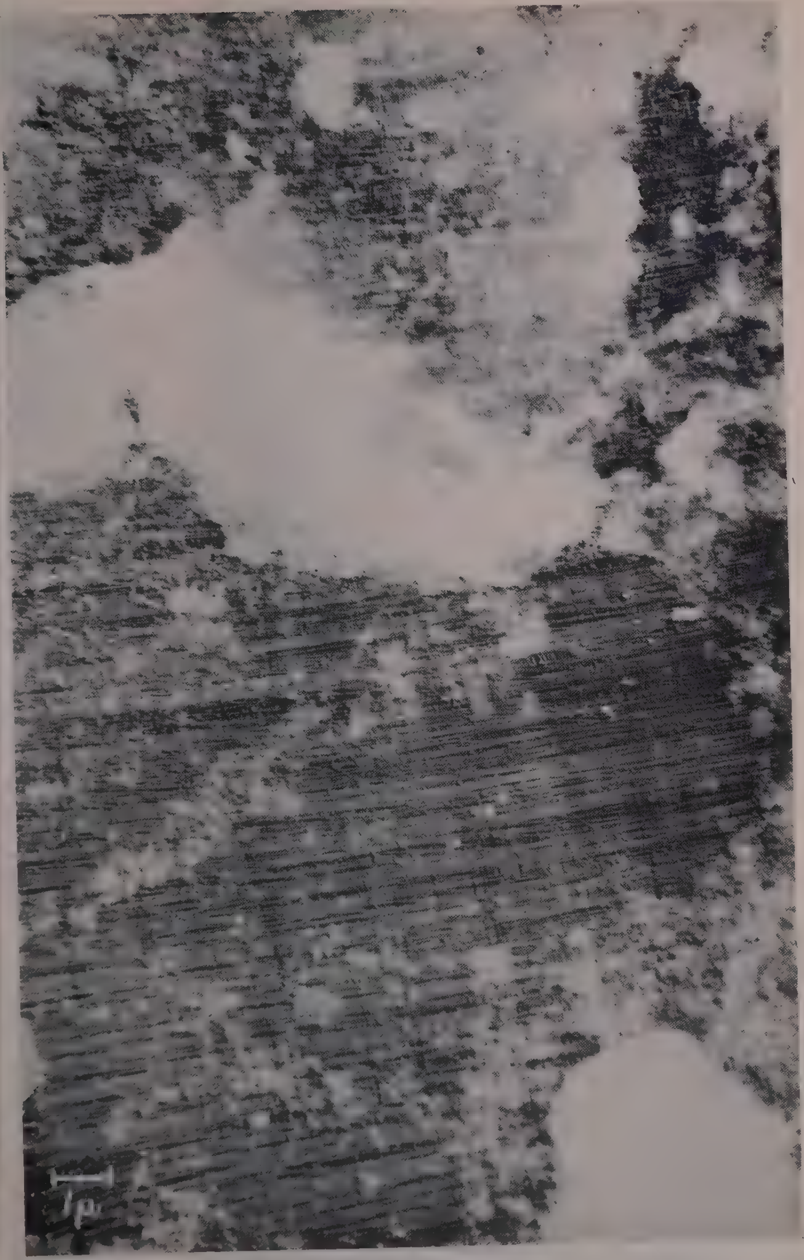

PLATE I. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.-Au. ×8,500

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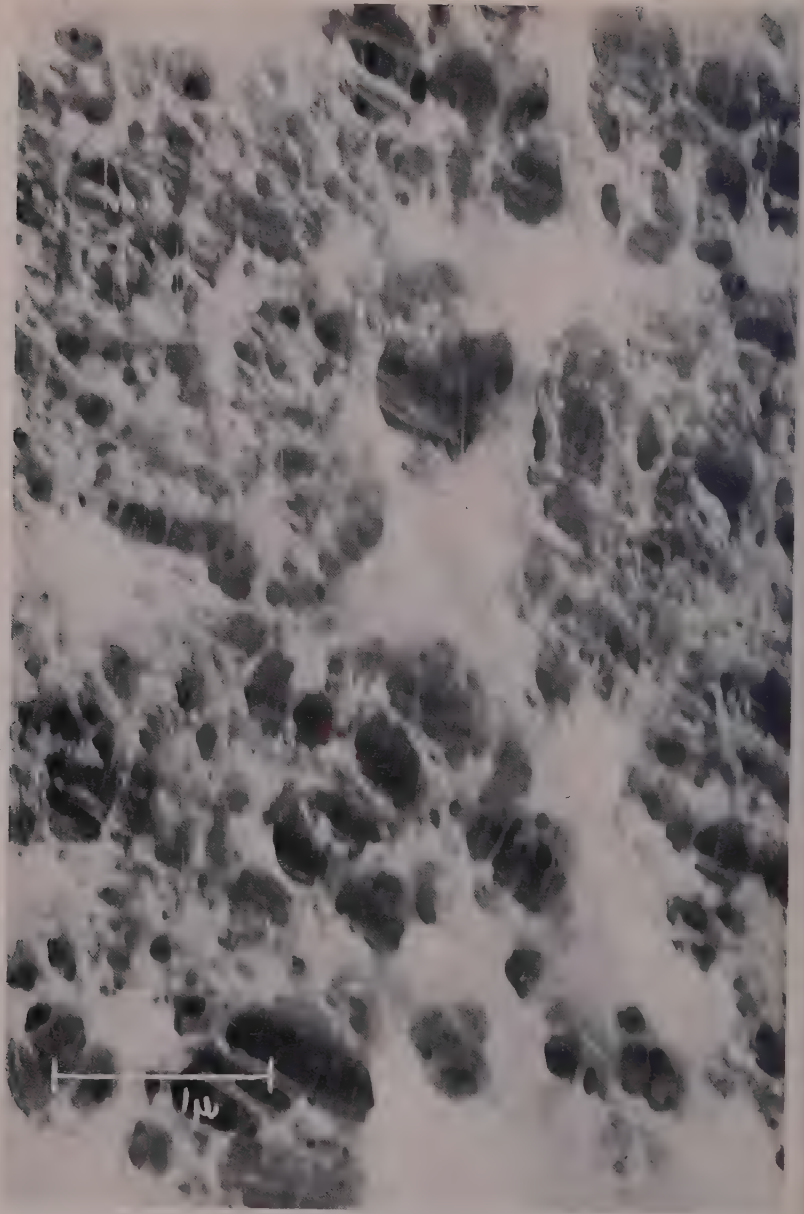
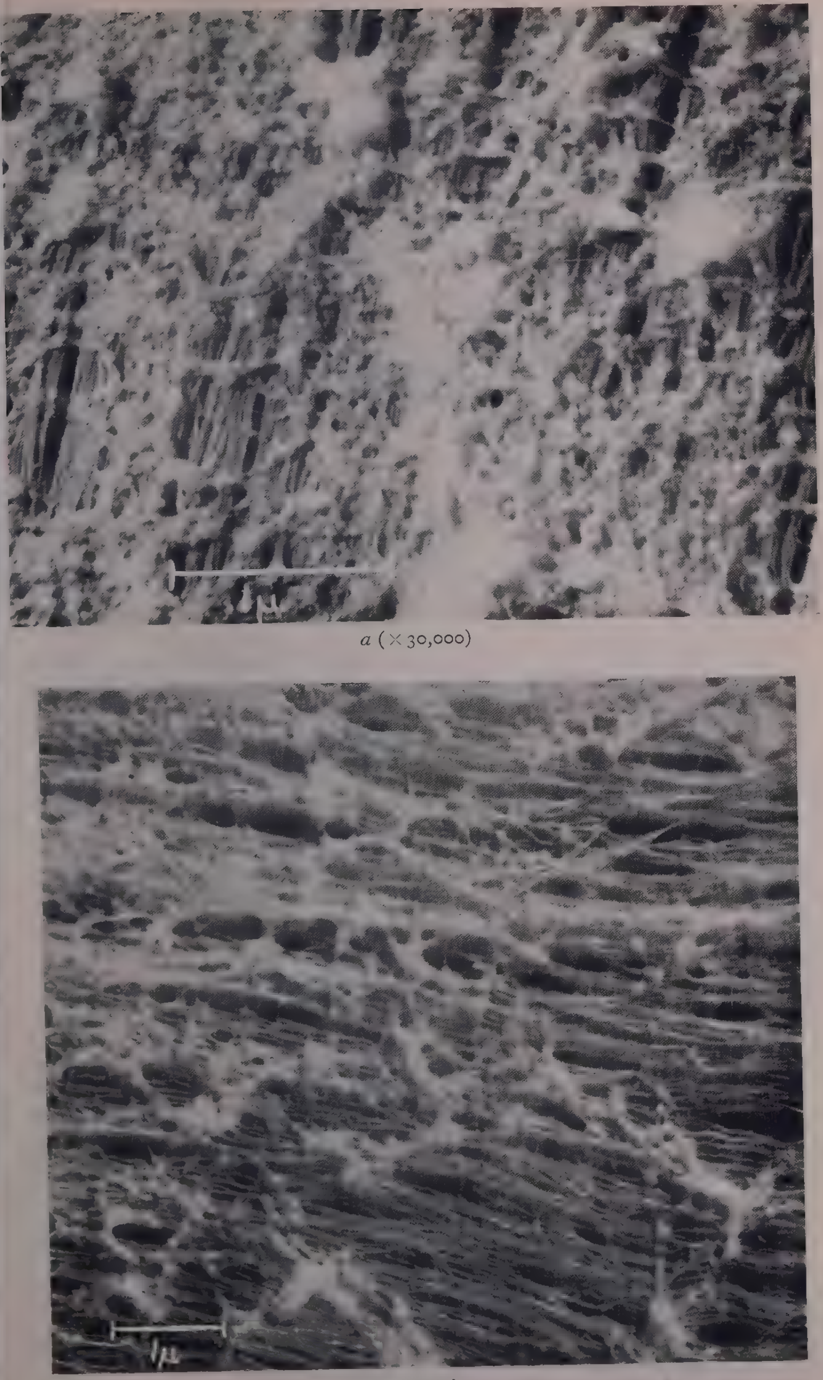


PLATE II. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.–Au. × 30,000

R. D. PRESTON, E. NICOLAI, AND B. KUYPER



a (× 30,000)

b (× 15,000)

PLATE III. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.–Au.

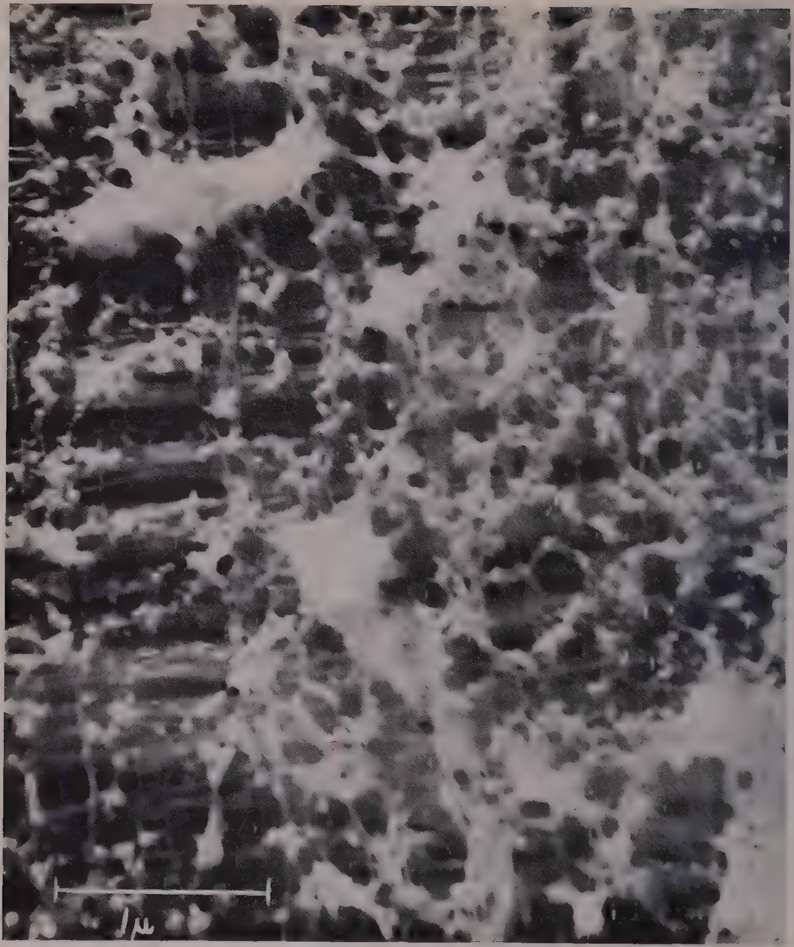


PLATE IV. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.–Au. × 30,000

R. D. PRESTON, E. NICOLAI, AND B. KUYPER

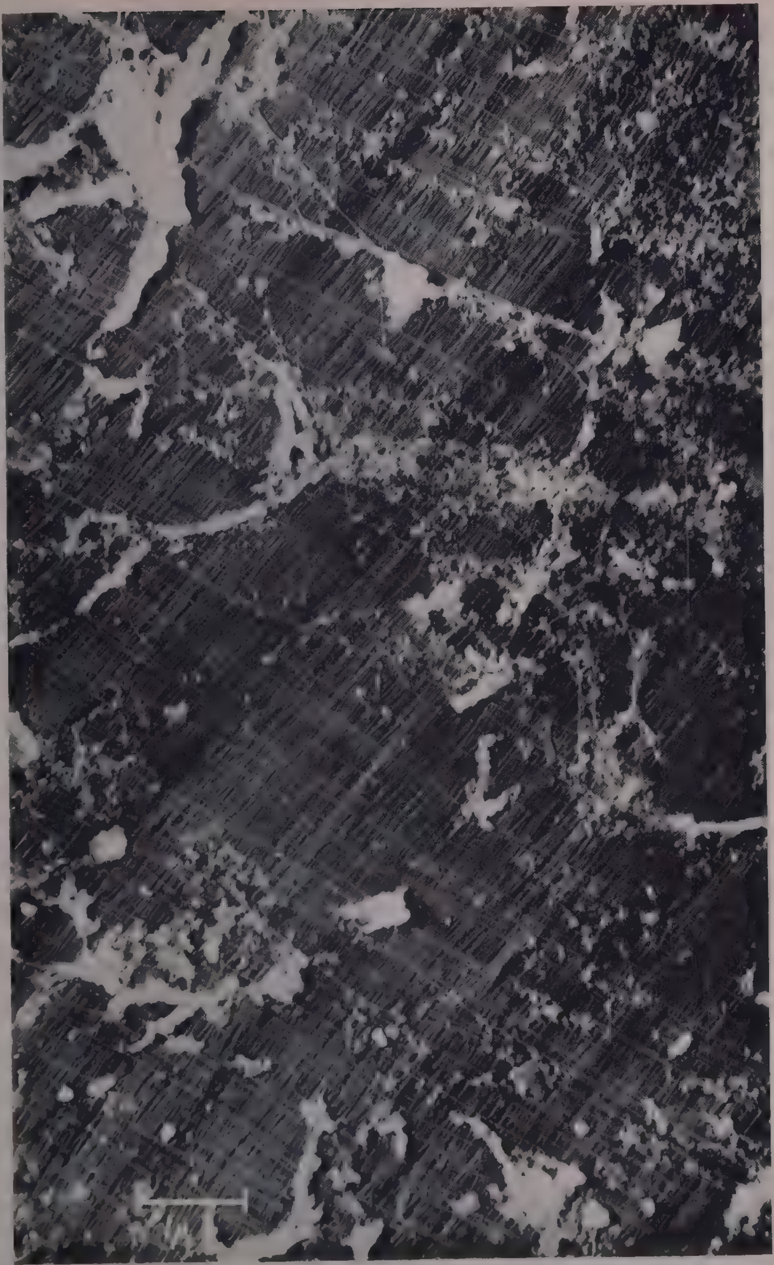


PLATE V. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.-Au. × 15,000

R. D. PRESTON, E. NICOLAI, AND B. KUYPER

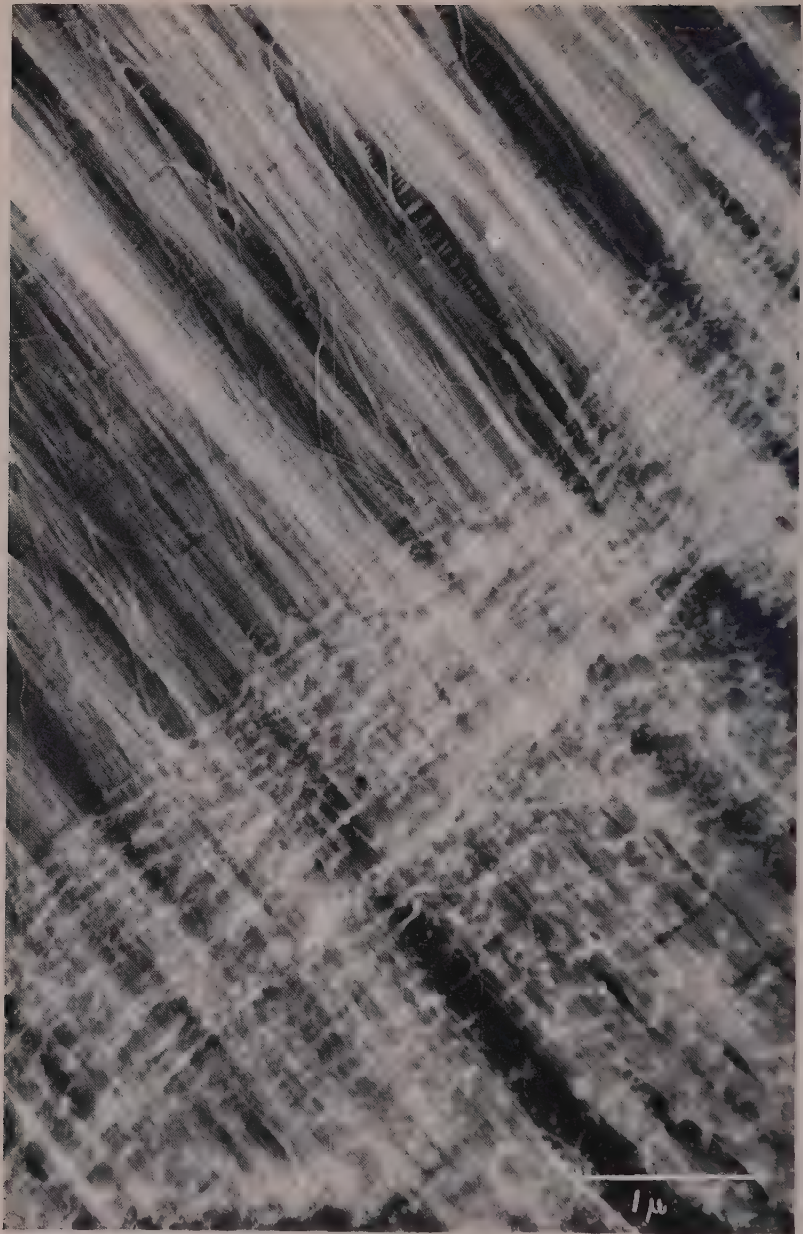


PLATE VI. Electron micrograph of the innermost surface of the walls of vesicles of *Valonia macrophysa*, taken from freeze-dried material. Shadowed with Pd.–Au. × 22,500

R. D. PRESTON, E. NICOLAI, AND B. KUYPER

e cytoplasm–wall association is lost (so that the cytoplasm to the upper ft of the area of alinement in Pl. VI has become detached during specimen reparation). The process must, however, be left in such vague and speculative terms until it becomes possible to examine larger quantities of material.

The authors wish to acknowledge the kindness shown to them by Professor ohrn and Dr. P. Dohrn both personally and in placing the facilities of the tazione Zoologica at their disposal. They are also indebted to Professor Ionroy for the freeze-drying of the material and to the British Association r the Advancement of Science for placing at their disposal the British ssociation Table at the Stazione. The senior author is indebted to the epartment of Scientific and Industrial Research for a grant in aid of a search programme of which this forms a part.

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The Inhibition of α-Amylase and Phosphatase Contaminants of Potato Phosphorylase Preparations

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SUMMARY

α-Amylase contaminating partially purified potato phosphorylase was inhibited by M/5,000 $HgCl_2$ without sensibly affecting phosphorylase activity. Phosphatase was simultaneously inhibited by M/50 NaF. The $HgCl_2$ prevented amylolysis of both added priming polysaccharide and synthesized amylose as shown by absence of maltose. At very low initial primer concentrations autocatalysis due to effective increase in primer concentration resulting from amylolysis was eliminated, but there was a rise in glucose production due to increased phosphatase action, and the ratio of glucose to phosphate produced was nearly 1·0. There was thus a marked interaction between phosphorylase and phosphatase activities, phosphatase being inhibited when high primer levels induced maximum initial phosphorylase activity.

When both $HgCl_2$ and NaF were added the phosphorylase reaction, at high primer concentration, proceeded to equilibrium accompanied by only a very small increase in reducing power, and no glucose or maltose could be detected. At low primer concentration the reaction proceeded extremely slowly, and ceased when less than 10 per cent. of the glucose-1-phosphate had been converted. Equilibrium at about 75 per cent. conversion of glucose-1-phosphate was reached with primer concentrations from 10 to 500 mg. per cent., and the calculated mean chain length of the amylose produced was from approximately 1,000 units down to 20. At primer concentrations below 10 mg. per cent. the reaction virtually ceased when the calculated mean chain length was about 800 units. The course of the reaction effected by potato phosphorylase when α-amylase and phosphatase were inhibited was thus analogous to that reported for crystalline muscle phosphorylase, except that the maximum chain length of the amylose was about 1,000 units as compared with 200 units for the muscle enzyme.

IT is recognized that removal of all traces of α-amylase from plant phosphorylase preparations, which are based on the original ammonium sulphate precipitation technique of Hanes (1940) or the modification introduced by Barker, Bourne, and Peat (1949), presents considerable difficulties (Barker, Bourne, Wilkinson, and Peat, 1950; Hobson, Whelan, and Peat, 1950). In consequence the course of the reaction, when purified potato phosphorylase preparations act upon glucose-1-phosphate (g-1-p) in the presence of very small amounts of priming polysaccharide, is autocatalytic because degradation by the α-amylase of synthesized polysaccharide increases the number of chain ends available to

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hich glucose can be transferred. Traces of α-amylase whose action upon arch can only be detected by increase of reducing power after many hours incubation are sufficient to affect the blue value of synthetic amylose ailey, Thomas, and Whelan, 1951). It is well known that the amylases are pecially sensitive to the inhibiting action of heavy metal salts, and the above thors have reported that concentrations of Hg sufficient to inhibit the amylase are almost without effect upon potato phosphorylase action, so that g may be used to prevent α-amylase action in preparations containing both nzymes.

The need for a preparation of potato phosphorylase exhibiting neither mylolytic nor phosphatase activity led to an investigation of the effects of gCl$_2$ and NaF as selective inhibitors of these two enzymes on the course of nylose synthesis from g-1-p by the phosphorylase of potato juice.

EXPERIMENTAL

Substrate. The g-1-p preparation used has already been described (Porter, 950).

Enzyme. Potato phosphorylase was prepared, with slight modification, by he method of Hanes (1940). One kilogram of potatoes was sliced and treated or 20 minutes with 0·5 per cent. sodium dithionite, then washed and the lices disintegrated in an Atomix blender. After filtration through muslin and entrifugation, the 600 ml. of juice obtained was adjusted to pH 7·0 with N. NaOH and fractionated once with $(NH_4)_2SO_4$. The fraction precipitating etween 30 and 50 per cent. saturation was collected and dialysed for 4 hours gainst 0·05M. citrate at pH 6·5. The opaque liquid, which contained by design some amylase and phosphatase as well as phosphorylase, was made to 0 ml. with citrate at pH 6·5 and kept at 3° C. The preparation had been stored or 3 months when used as enzyme in these experiments. Hydrolysis for 7 minutes with N. H_2SO_4, of aliquots of the reaction mixtures containing g-1-p detailed below, showed that after 24 hours of incubation all the phosphate was present either as g-1-p or as free phosphate (P). If any phosphoglucomutase was originally present therefore, either it became inactivated during he storage period or else any 6-ester produced was readily split by the hosphatase.

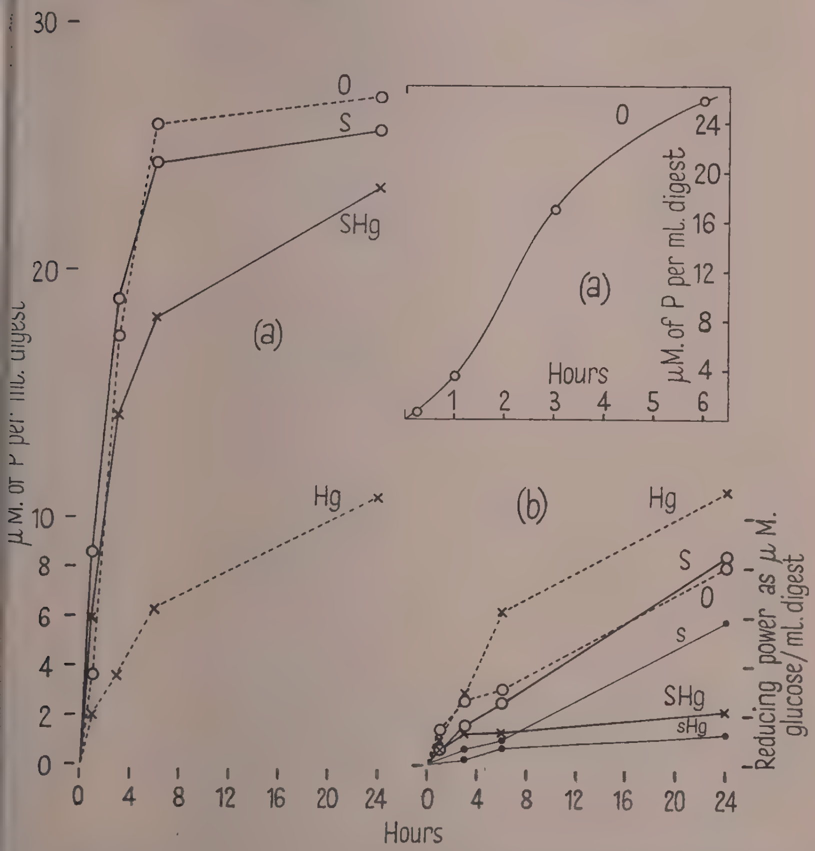
Primer for phosphorylase reaction. Soluble starch prepared from commercial potato starch by the method of Hanes and Cattle (1938) was used.

Measurement of enzyme activity. The enzyme reactions were carried out at 30° C. in 0·05 M. citrate at pH 6·5. The digests had a total volume of 3 ml. and contained in addition to buffer 0·033 M. g-1-p and 0·5 ml. of phosphorylase preparation. When required 15 mg. of soluble starch was included to produce an optimal priming effect, and the digests were made M/5,000 with respect to $HgCl_2$ for inhibition of α-amylase and M/50 with respect to NaF for inhibition of phosphatase. The course of the reaction was followed by measuring release of P from g-1-p and increase in reducing power. P was estimated by the method of Fiske and Subbarow (1925) in aliquots of the digest as already described (Porter, 1950). Reducing power was determine by the procedure of Nelson (1944) using a copper reagent modified for smal amounts of sugar (private communication from Dr. Somogyi). 0·1 ml. o digest was delivered into 0·9 ml. of water and 1 ml. of copper reagent, and the estimation carried out immediately.

Colorimetric measurements were made using the Evans Electroselenium (EEL) photo-electric colorimeter, with a red filter (Ilford 608, transmission 650 to 700 mμ) for the phosphate determination and a green filter (Ilford 625, transmission 530 to 550 mμ) for the sugar determination. Results are expressed as micromoles of phosphate and apparent glucose produced. The reducing sugars arising from amylase or phosphatase action, namely maltose and glucose, were identified on paper chromatograms using butanol/acetic acid as the developing solvent and spraying with benzidine trichloracetic acid (Bacon and Edelman, 1951). The iodine stains of the polysaccharide formed were noted by spraying the starting lines of the papers with 0·2 per cent. (w/v) I_2 in 2 per cent. (w/v) KI and by adding iodine to aliquots of the digests.

Results. The effect of $HgCl_2$ at optimal and at very low primer concentrations on the breakdown of g-1-p by the potato preparation is shown graphically in Fig. 1. The digests contained traces of priming polysaccharide probably in the g-1-p, producing the condition of very low primer concentration, and optimal primer concentration was obtained by adding 500 mg. per cent. of soluble starch. At the optimal primer concentration and without Hg the release of P (curve *S*, Fig. 1*a*) followed a course usual in phosphorylase action, reaching an equilibrium value in about 6 hours. Simultaneously there was an increase in reducing power (curve *S*, Fig. 1*b*) which when calculated as glucose was equivalent to about 30 per cent. of the P produced at the final equilibrium level. This reducing power derived in the main from the secondary action of the α-amylase upon the polysaccharides, as indicated by the fact that increase was approximately linear for 24 hours while P release was nearly at the equilibrium value after 6 hours. Furthermore, a linear increase in reducing power resulted from the action of the enzyme preparation upon the starch primer when g-1-p was absent and this effect was not apparent when Hg was added (curves *s* and *sHg*, Fig. 1*b*). At the very low primer concentration P release followed an autocatalytic course, being slow for the first half hour and then increasing rapidly (curve *O* with inset of first 6 hours of reaction, Fig. 1*a*). Increase in reducing power was again approximately linear for most of the 24-hour reaction time (curve *O*, Fig. 1*b*), but during the first hour the rate of increase was higher than at the optimal primer level, and the ratio of apparent glucose to P produced was 40 per cent. This high ratio suggests that phosphatase action accounted for much of the P liberated in the early stage of the reaction. The total P after 24 hours was a little higher than when primer level was optimal.

The presence of M/5,000 Hg in the digests diminished only a little the liberation of P at the optimal primer level (curve *SHg*, Fig. 1*a*), but there was a considerable effect at the low primer level (curve *Hg*, Fig. 1*a*); moreover curve was no longer autocatalytic, since inhibition of the α-amylase pre-nted production of additional primer during the reaction. Conversely pro-ction of reducing power was depressed at the high primer level as secondary



IG. 1. Effect of $HgCl_2$ on the action of partially purified potato phosphorylase upon lucose-1-phosphate at optimal and very low primer concentrations. (*a*) Release of free hosphate; (*b*) increase in reducing power. Digests at 30° C. 3 ml. containing 0·033M. -1-p; 0·05M. citrate, pH 6·5; 0·5 ml. enzyme; and either 500 mg. % soluble starch (curves and *SHg*) or no added primer (curves *O* and *Hg*); also either M/5,000 $HgCl_2$ (curves *Hg* and *Hg*) or no $HgCl_2$ (curves *S* and *O*). Digests containing 500 mg. % soluble starch, ith and without M/5,000 $HgCl_2$ but omitting g-1-p (curves *s* and *sHg*, b). Inset: course of reaction for first 6 hours (curve *O*, *a*).

reakdown of the polysaccharide was prevented, but it was much enhanced t the low primer level (curves *SHg* and *Hg*, Fig. 1*b*). In this latter case the atio of apparent glucose to P produced was throughout nearly 1·0, so that ere the major reaction was that of phosphatase acting directly or indirectly pon g-1-p. It is therefore evident that phosphatase activity increased at low primer concentration to a marked extent when increase in phosphorylas activity during the reaction period was prevented by the presence of Hg (c curves *O* and *Hg*, Fig. 1*b*).

After 24 hours' incubation aliquots of the digests were examined for thei carbohydrate constituents on paper chromatograms. Digests not containin Hg all showed the presence of both glucose and maltose, while maltose wa absent from those to which Hg had been added, which is taken to prove tha α-amylase had been effectively inhibited by this treatment. In the diges containing Hg, at low primer concentration, the glucose spot was much mor intense than in the other cases (cf. curve *Hg*, Fig. 1*b*), thus confirming th conclusion that the high reducing power arising under these conditions wa the result of glucose production by phosphatase action.

Spraying the starting-line of the paper with iodine showed that all th digests contained iodine-staining polysaccharide. At the low primer concentration the iodine stains were blue. In the absence of Hg the spot was rathe faint, indicating that much of the synthesized polysaccharide had been degraded by α-amylase; in the presence of Hg the spot was small but the colou more intense, proving that slow phosphorylase action had proceeded leadin to formation of a small amount of polysaccharide with a relatively long mea chain length. At high primer concentration the iodine stains were purple. Calculation of the mean chain length of the polysaccharide, based on the end-group value for the amylopectin constituent of the added starch (4·5 per cent.), would give at the reaction equilibrium an approximate value of 20 units. This low value would account for the observed purple stain, the minimum chain length producing a blue stain being about 30 units (Swanson, 1948).

The effect of adding NaF in addition to $HgCl_2$ on the course of the reaction is shown in Fig. 2. At the optimal primer concentration the P released after 24 hours was a little lower in the presence of the fluoride (curves *S* and *SF*, Fig. 2*a*), but when primer concentration was low and, as already noted, phosphate release largely due to phosphatase action, the addition of fluoride by inhibiting the phosphatase reduced the liberation of P to an extremely low level (curves *O* and *F*, Fig. 2*a*). When the optimal amount of primer was present the relatively low reducing power produced in the presence of Hg was still further diminished by the addition of fluoride (curves *S* and *SF*, Fig. 2*b*), and at low primer level the relatively high reducing power was also largely suppressed (curves *O* and *F*, Fig. 2*b*), thus confirming again that the high value resulted from phosphatase action, and also that the amount of phosphatase action depended upon the initial phosphorylase activity.

Examination of the digests as before for their carbohydrate constituents showed that no maltose was present. Glucose again appeared in quantity at the low primer level in the absence of fluoride (cf. curve *O*, Fig. 2*b*), but in traces only at the high level. In the presence of fluoride traces of glucose stil appeared at the low level, indicating the phosphatase inhibition was not quite complete, but at the high level none was detectable, so that the very smal increase of reducing power (curve *SF*, Fig. 2*b*) can be attributed to the polyccharide formed. Spraying the starting-line of the chromatogram with dine again showed blue staining polysaccharides at the low primer level and urple staining at the high level. It is of interest that at the low primer level

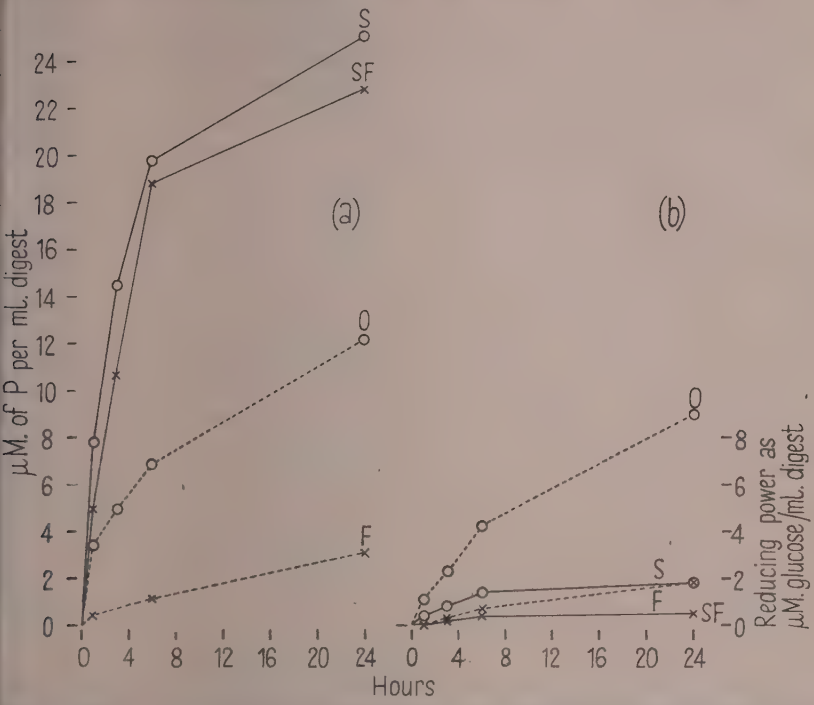


FIG. 2. Effect of $HgCl_2$ together with NaF on the action of partially purified potato phosphorylase upon g-1-p at optimal and very low primer concentrations. (*a*) Release of free phosphate; (*b*) increase in reducing power. Digests at 30° C. as detailed for Fig. 1, but all containing M/5,000 $HgCl_2$ and either 500 mg. % soluble starch (curves *S* and *SF*) or no added primer (curves *O* and *F*) and also either M/50 NaF (curves *SF* and *F*) or no NaF (curves *S* and *O*).

when both α-amylase and phosphatase were inhibited, and both P release and reducing power increase were very small, the extremely small phosphorylase activity could readily be detected by the appearance of the blue iodine stain on the paper.

The effect of a number of different primer concentrations on the reaction when both α-amylase and phosphatase were inhibited is shown in Fig. 3. There was no indication of autocatalysis and when no primer was added P release was very small. For primer concentrations from 500 to 10 mg. per cent. the reaction proceeded to an equilibrium point corresponding to about 75 per cent. conversion of g-1-p. The calculated chain lengths of the polysaccharides produced, based as before upon the end groups of amylopectin, are from approximately 20 to 1,000 units. Below a primer concentration of

10 mg. per cent., the reaction practically stopped after about 3 hours, althoug on prolonged incubation (24 hours) it was clear that phosphate continued to liberated very slowly, probably by the small trace of phosphatase remainin The calculated chain lengths at the end of 3 hours were of the order of 8 units, for the low primer levels, so that a value of this order appears to be th maximum for potato phosphorylase action. Full phosphorylase activity coul

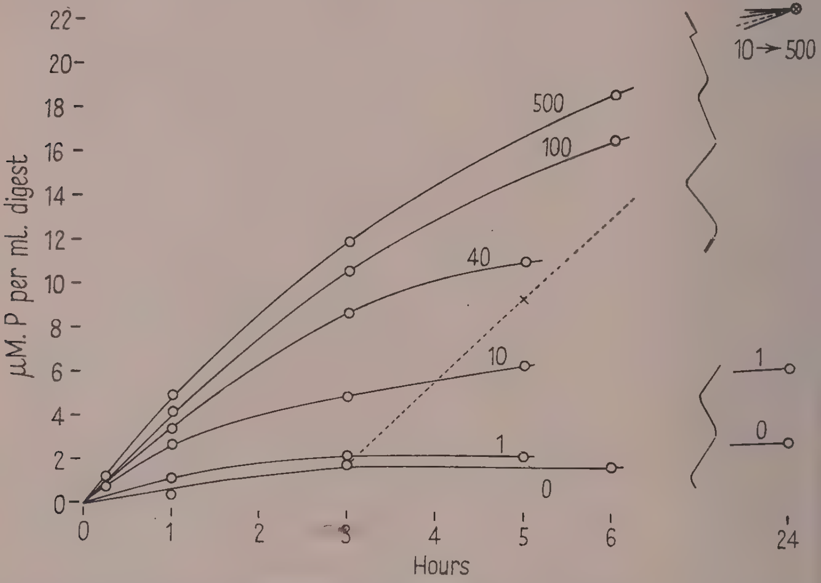


FIG. 3. Effect of primer concentration on the action of partially purified potato phosphory- lase upon g-1-p when α-amylase and phosphatase contaminants are inhibited. Digests at 30 3 ml. containing 0·033M. g-1-p; 0·05M. citrate, pH 6·5; 0·05 ml. enzyme, M/5,000 HgCl$_2$ M/50 NaF and mg. % soluble starch as marked on curves. Dotted line shows effect of addin 500 mg. % soluble starch after 3 hours to a digest without added primer.

be demonstrated at any time by adding primer as illustrated by the dotte line in Fig. 3, where primer was added after 3 hours to a duplicate digest. Al the digests stained blue with iodine after 24 hours, except that containing 50 mg. per cent. of primer which as before stained iodine purple.

The family of curves obtained is similar to that found by Cori, Swanson, and Cori (1945) for crystalline muscle phosphorylase.

DISCUSSION

Cori, Swanson, and Cori (1945) have shown that the course of the reaction when crystalline muscle phosphorylase acts upon g-1-p depends upon the concentration of priming polysaccharide added. When optimal amounts of primer are present the reaction is unimolecular and proceeds to an equilibrium, whose exact value depends upon the pH, corresponding to about 75 per cent. conversion of the ester to amylose. As primer concentration is reduced the reaction departs from the first order form and at very low primer concentrations ceases before true equilibrium is reached; cessation occurs when the mean chain length of the amylose reaches about 200 units. When chemically instead of biologically synthesized g-1-p is used, to ensure absence of priming molecules, no reaction occurs.

Potato phosphorylase has not so far been obtained crystalline and even after repeated fractionations with $(NH_4)_2SO_4$ the phosphorylase reaction proceeds, at very low primer concentrations, in an autocatalytic manner, indicating continuous increase during the reaction of chain ends to which glucose can be transferred. This type of reaction is observed although the presence of α-amylase, which may bring about such a situation, cannot apparently be detected by the usual means (Katz and Hassid, 1951); but when special attention was paid to removal of all traces of priming material, by pretreatment with salivary α-amylase, these authors found that no phosphorylase action occurred at least for 24 hours. It has now been shown that when α-amylase was effectively inhibited, by the selective action of Hg, at very low primer levels, the reaction was no longer autocatalytic and that phosphorylase activity remained extremely low. Autocatalysis when using even highly purified potato phosphorylase preparations must therefore be attributed to failure to remove completely the α-amylase, and it is evident that extremely low α-amylase activity is sufficient to initiate an autocatalytic type of reaction, as well as affecting the blue value of the amylose.

Small amounts of phosphatase may also modify the course of the reaction. In the virtual absence of phosphorylase action, brought about by the very low primer concentration, and inhibition of α-amylase by Hg, phosphatase activity is manifested by an almost equimolar production of glucose and phosphate, but when primer is added phosphorylase competes successfully for the g-1-p and glucose production is reduced to a very low level. When the equilibrium level for phosphorylase action has been reached, however, continued slow release of P still occurs, and it is clear that slow hydrolysis of g-1-p by phosphatase may bring about reversal of the reaction and breakdown of amylose. In these circumstances the final reaction products would be glucose and phosphate, but the reverse reaction is liable to be very slow owing to further inhibition of the phosphatase by P.

When fluoride was added as well as Hg so that both α-amylase and phosphatase were inhibited, the system obtained from the potato preparation behaved in a manner similar to that recorded for the crystalline muscle enzyme at all primer levels tested. It may therefore be concluded that under these conditions the release of P is derived solely from phosphorylase activity. The reaction apparently only differed from that of the muscle enzyme in that the maximum mean chain length of the synthetic polysaccharide was calculated to be of the order of 1,000 units for the potato enzyme as compared with 200 units for the muscle enzyme (Cori, Swanson, and Cori, loc. cit.).

It is perhaps of interest that the presence of quite small amounts of contaminating proteins which can act upon the components of the phosphorylase system may profoundly alter the course of the reaction and indeed determine whether or no synthesis can occur. Whether such interactions between proteins will prove to have biological significance must be a matter for speculation at present, but the interactions between α-amylase, phosphatase, and phosphorylase activities is a good example of the now familiar phenomenon of the effect of one protein on the enzyme activity of another.

I am grateful to my colleagues, Dr. J. Edelman for running the paper chromatograms and Mr. R. V. Martin for help with the analyses.

Note. Since this paper was written Gilbert and Patrick (1952) have described a preparation of potato phosphorylase by ethanol precipitation in which no amylase or phosphatase could be detected.

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The Use of Radioactive Iodine in the Study of a Plant Growth-Regulator

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SUMMARY

2-iodo-4-chlorophenoxyacetic acid (ICPA) labelled with I^{131} was applied in the form of a 0·25 per cent. olive oil solution (using 2·5 per cent. ethyl alcohol as accessory solvent) to the upper leaf surface of young broad bean and oat plants at the rate of 0·01 ml. solution per plant. The initial activity of the solution was 3·75 mc. per ml. Epinastic effects were induced in the bean but not in the oat. The movement of the I^{131} within the plant was followed by means of autoradiographs. Results indicate rapid absorption and translocation of a high proportion of the applied material in the broad bean, radioactivity appearing in quantity in every part of the plant within 27 hours of treatment, with a marked concentration at the upper end of the stem and another at the lower end of the stem and top of the tap root. In the oat, on the other hand, absorption and translocation were much slower; only under the most favourable conditions did a small amount of of I^{131} reach the root in 27 hours, and the amount absorbed was much less than in the bean. There was no upward movement of the I^{131} into untreated leaves of the oat.

INTRODUCTION

THE herbicidal action of the plant growth-regulators 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2:4-dichlorophenoxyacetic acid (2,4-D) and their selective effects have now been known for 10 years. Knowledge of the mode of action of these compounds and of the mechanism of their selectivity is, however, still very limited.

The introduction of radioactive isotopes offered a new means of investigation which seemed particularly suited to problems concerned with the entry into and movement within the plant of growth-regulators and related substances.

Holley, Boyle, and Hand (1950), and Fang and his co-workers (1951) have given some indication of the movement and distribution of C^{14}-labelled 2,4-D in the broad bean, while Mitchell and Linder (1950) have carried out similar work using 2:4-dichloro-5-iodo-phenoxyacetic acid labelled with I^{131}. None of these workers has considered the problems of absorption and translocation in resistant species of plant, though Mitchell *et al.* (1947) and Wood, Mitchell, and Irving (1947) had studied the movement and effects of 2-iodo-3-nitrobenzoic acid, labelled with I^{131}, in oat as well as in bean plants. The action of this compound, however, appears to differ from that of the phenoxyacetic groups of compounds.

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In the present investigation, autoradiographic methods were used to follow the progress of 2-iodo-4-chlorophenoxyacetic acid (ICPA), labelled with I^{131}, in broad bean and oat plants. The amount of the compound absorbed by the plant was believed to be very small; probably too small to be satisfactorily measured by chemical means. In any case, chemical measurement of phenoxyacetic compounds is a long and difficult process, not suitable for the micro-scale methods which would have to be employed and not altogether reliable.

MATERIAL AND METHODS

1. *Selection of a suitable compound.* A series of preliminary trials was carried out to compare, in seedling root-growth-reduction tests in agar and on growing plants, the effects of the three iodine derivatives of the phenoxyacetic acid series reported to exert a growth-regulatory action, viz. 4-iodo-phenoxyacetic acid, 2:4-dichloro-5-iodo-phenoxyacetic acid, and 2-iodo-4-chloro-phenoxyacetic acid. Results showed the marked superiority of the last-named over the other compounds, in both growth-reducing and epinastic effects. Further trials showed that the most suitable formulation was an olive oil solution containing 0·1–0·5 per cent. ICPA. Higher concentrations caused damage to the treated leaves, while at lower concentrations the epinastic effect was unreliable. In the preparation of this solution it was found necessary to use an intermediate solvent since crystalline ICPA would not dissolve directly in olive oil. The final formulation therefore contained 0·25 per cent. ICPA and 2·5 per cent. ethanol in olive oil. This produced epinastic effects in under 24 hours with a minimum of damage to the treated leaves. The same formulation, using the radioactive form of the compound (I^{131} CPA) was used in all the experiments described below. An olive oil/alcohol mixture, in the same proportions but without ICPA, had no effect on the plants, causing neither damage nor epinasty. The preparation of the radioactive material was carried out by R. L. Jones (1952) of I.C.I. (Dyestuffs Division) Research Department, Blackley, Manchester.

The initial activity of the solution (150 mc. in 40 ml., i.e. 3·75 mc. per ml.) was determined by the amount of activity believed necessary to produce satisfactory autoradiographs and by the assumption that only a small proportion (*c.* 1 per cent.) of the radioactive material would enter the plant. Activity was limited by technical difficulties in the preparation of the radioactive ICPA.

I^{131} was supplied by the Atomic Energy Research Establishment at Harwell, in the form of a solution containing NaI^{131} in water. The half-life of I^{131} is 8 days.

2. *Experimental methods.* Broad bean and oat plants were grown in sand and supplied with a nutrient solution.

The broad beans were treated when 6–12 in. high (with 3–6 true leaves) and oats at the 2- or 3-leaf stage (height 4–8 in.). The substance was applied to the upper leaf surface in both cases. Oat leaves were held down by wire frames, to keep the treated part of the leaf in a horizontal position and prevent the solution running off the leaf tip or down towards the stem.

The radioactive solution was applied by means of a remote-control pipette based on the 'remote control applicator' of Mitchell and Linder (1950), which delivered a standard dose of 0·01 ml.

At various intervals after treatment plants were cut up and portions mounted on slabs of 'Plasticine' contained in rectangular steel dishes, covered with the X-ray film, a sheet of cardboard, and a steel lid. The latter was sufficiently heavy to hold the film in close contact with the plant tissue. Standard exposure time was 24 hours.

Kodak 'Crystallex' X-ray film was used, developed in D 19*b* for 5 minutes at 65° F., and fixed in a normal acid-fixing bath.

Precautions against the health hazard. In view of the high degree of radioactivity of the material employed, and because the emitted radiation consisted n part of gamma-rays, it was necessary to take the most stringent precautions against the health hazard. These precautions were bases on the 'Introductory Manual on the Control of Health Hazards from Radioactive Materials, Issue No. 2', published by the Ministry of Supply, Atomic Energy Research Establishment.

RESULTS

The movement of the I^{131} in the plant is illustrated by Figs. 1 -5 modified from autoradiographs.

In the interpretation of results, two factors must be considered:

1. The distribution of I^{131} is not necessarily the same as that of the I^{131} CPA originally applied. Decomposition, or transformation of the ICPA molecules into other compounds, may have taken place within the plant.
2. Artefacts, in the form of 'pressure-marks' and stains, occurred on many autoradiograph films. The stains are due probably to reducing agents (e.g. catechols) present in the plant sap, acting on the photographic emulsion. In all cases, therefore, films were compared with controls made from untreated plants, and only images not found on control films were regarded as valid. In later experiments stains were eliminated by interposing a thin sheet of 'Alkathene' (0·002 in. thick) between the plant material and the film. 'Pressure-marks' also were reduced by this method, but there was some loss of definition in the image.

Films made from bean plants 2 hours after treatment showed that some of the radioactive compound had entered the treated leaf and been translocated down into the petiole; in the oat there was some downward movement in the treated leaf.

Eight hours after treatment, considerable movement had taken place in the bean, I^{131} now being present throughout the main stem with a slight accumulation near the apex (Fig. 1). Less movement had taken place in the oat, radioactivity being present only in the treated leaf, as far down as its attachment to the stem (i.e. to the region of attachment of the seed). (Fig. 4.)

Sixteen hours after treatment the compound was present in every part of

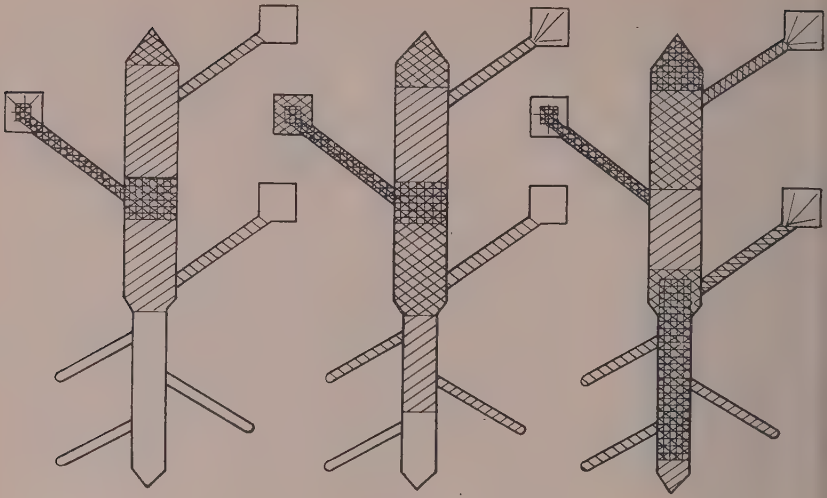

Fig. 1. Fig. 2. Fig. 3.

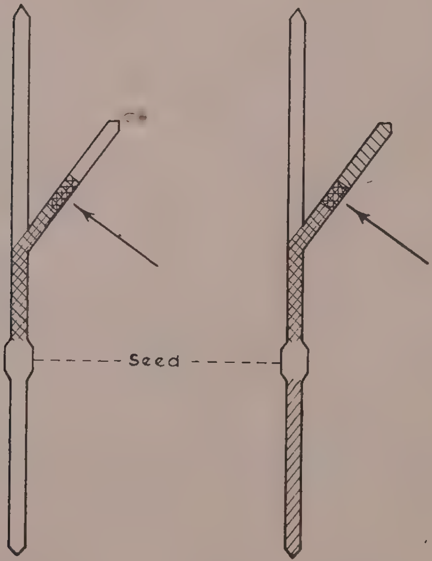

Fig. 4. Fig. 5.

Figs. 1–5. Diagrams, taken from autoradiographs, to show the movement of I^{131} (applied as I^{131}CPA to leaves) in broad bean and oat plants. The depth of shading indicates the amount of I^{131} present, as shown on the autoradiograph plates. Fig. 1, 8 hours; 2, 16 hours; 3, 27 hours Broad bean. Fig. 4, 8 hours; 5, 27 hours Oat. The treated leaf on the bean was always the left-hand leaf. The arrow indicates the point of application on the oat.

e bean plant except the lowest roots and untreated leaves below the treated ne (Fig. 2); there was no further movement in the oat.

Greatest distribution of I^{131}CPA in the bean was reached 27 hours after eatment, when the compound was to be found in all parts of the plant, with marked accumulation at the top and bottom of the stem and in the upper art of the tap-root (Fig. 3).

Under the most favourable growing conditions a small amount of I^{131} ould now be detected in the roots of the oat (Fig. 5). There was no upward ovement into untreated oat leaves, now or at any time up to 6 days after eatment, the maximum at which autoradiographs were made. Entry of I^{131} nto the oat root was often delayed until 2 or more days after treatment.

Further changes took place in the bean, notably a decrease in the amount of ^{31}CPA in the treated leaf, in the period 1–6 days after treatment. The concentration of the compound in the middle region of the stem also decreased, ut there was no obvious increased accumulation in other parts of the plant.

DISCUSSION

The selective action of growth-regulators has been the subject of much eculation. There is some evidence on the mode of action of these compounds susceptible species, but very little as to why they are relatively inactive gainst resistant species. Several theories have been put forward to explain sistance, e.g. (i) that the compounds are unable to enter the plant; (ii) that, aving entered the plant, their movement is obstructed, or (iii) their action is hibited chemically; (iv) that resistant species lack certain enzyme systems fected by growth-substances, which are present in, and vital to, plants of usceptible species.

Results of the present study tend to favour theories (i) and (ii), i.e. entry ICPA into the resistant plant (in this case, oat) and subsequent movement ithin it, are both obstructed, though neither is completely inhibited. No idence is offered on the question of chemical inhibition.

The use in these experiments of an olive oil solution of ICPA instead of the queous solution more generally used in the field and in other laboratory ork might be held to impose a somewhat different set of conditions on the ntry of the compound into the plant. Response of the plant to treatment was, owever, shown in the preliminary trials to be the same in both cases; treatment with oil solutions gave less variation in response than that with aqueous lutions.

The effect of obstruction to entry and movement of the compound might ell be to prevent its concentration reaching the threshold of activity in the ssues in which it is active. This is supported by the fact that even plants of ighly resistant species are affected by very large doses of growth-regulators, pparently in a manner similar to that in which susceptible species are affected y more moderate doses. Thus the present work suggests that, in the case of CPA applied to oat plants, the origin of resistance may well be in physical or physico-chemical factors, rather than in purely chemical or enzymat inhibition of activity. There is, however, nothing in this work to preclude th latter operating as an additional factor in bringing about resistance.

CONCLUSIONS

I^{131}CPA is rapidly absorbed and translocated in the broad bean, reachin all parts of the plant (except the lowest root and lower untreated leaves) i 16 hours, and appearing everywhere within 27 hours. A high proportion o the material applied is absorbed.

In the oat a much smaller amount of the radioactive compound is absorbe and after a fairly rapid initial movement down the treated leaf, translocatio is much slower than in the bean, limited activity appearing in the roots in 2 hours only under the most favourable conditions.

ACKNOWLEDGEMENTS

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Metal Interrelationships in Plant Nutrition

I. EFFECTS OF SOME METAL TOXICITIES ON SUGAR BEET, TOMATO, OAT, POTATO, AND MARROWSTEM KALE GROWN IN SAND CULTURE

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SUMMARY

1. Sugar beet, tomato, potato, oat, and kale were grown in sand cultures with additions of several 'heavy' metals including Cr, Mn, Co, Ni, Cu, Zn, Pb, Cd, V, Mo in equivalent concentrations.

2. In sugar beet Cu^{++}, Co^{++}, Cd^{++} were usually highly active in causing chlorosis mainly suggestive of iron deficiency. The effect of Cr depended on valency and was greater as CrO$_4^{--}$. Zn^{++}, VO$_3^{-}$, Cr^{+++}, Mn^{++}, and Pb^{++} were less active in order.

3. The visual responses to Co^{++} and Ni^{++} varied greatly with the crop tested. Cu^{++}, however, always induced typical iron deficiency. Crop susceptibility also varied greatly. For example, Cu^{++} readily caused chlorosis in beet and also in tomato, and potato, but not in oat and kale.

4. Ni^{++} induced symptoms resembling manganese deficiency in potato and tomato and unusual oblique white and green banding leaves of oat. Zn^{++} induced apparent manganese deficiency in sugar beet and Co^{++} toxicity in tomato initially resembled manganese deficiency. Ni^{++} and Co^{++} were the most toxic of the metals tested.

THIS work arose partly from observations of the effects of metal-containing dustrial sewage and naturally occurring soil toxicities (e.g. zinc) on field ops and from the conclusions of Somers and Shive (1942) regarding the lationship between manganese toxicity and iron deficiency.

The ability of manganese to induce iron deficiency in certain plants has ceived considerable attention. Several instances were reviewed by Hopkins *al.* (1944), Wallace and Hewitt (1946), and Twyman (1946). Susceptible ants include widely separated types such as oat, pineapple, strawberry, and ya bean. Manganese, however, is not unique in this respect, and several eavy metals (at lower concentrations) may also induce chlorosis, visually ten resembling iron deficiency.

Numerous isolated examples in culture experiments or in the field include e effects of chromium, copper, zinc, cobalt, and nickel reviewed by Wallace d Hewitt (1946), Millikan (1947), and Hewitt (1948*a*). Where injury from dustrial sewage has occurred, the specific metals involved have not been entified. Spectrographic analyses[1] of plant material indicated the presence

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[1] These were made by Dr. R. L. Mitchell of the Macaulay Institute for Soil Research, berdeen, whose help is gratefully acknowledged.

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often simultaneously of copper, zinc, lead, titanium, nickel, manganese, an other metals in relatively high concentrations, but none were consistentl present in relation to the degree of injury observed.

This first paper deals with the general effects of toxic concentrations o several 'heavy' metals on plants grown in sand culture during 1947–9 and thei relation to iron and manganese nutrition. Interactions with molybdenum an nitrogen supply will be described later. Results of a preliminary experimen with sugar beet were reported by Hewitt (1948*a*).

EXPERIMENTAL

Methods. The plants were grown in bitumen-coated 10-in. clay pots wit a central drainage hole. Sand was retained by a watch-glass and glass woo which allowed free drainage. The sand was purified in the apparatus describe by Hewitt (1947). Rain-water was used to prepare nutrient solutions and wa purified by passage through Permutit ion-exchange resins which removed a great part of the mineral impurities. The residual iron and manganese level ranged between 0·002 and 0·006 p.p.m. and did not interfere. The basa nutrient solutions used in 1947 and 1948 had the following composition a m.eq./l.: KNO_3 3·33; $Ca(NO_3)_2$ 6·67; $MgSO_4$ 3·0; NaH_2PO_4 4·0; $CuSO_4$ 0·002; $ZnSO_4$ 0·002; $MnSO_4$ 0·02; H_3BO_3 0·09; $(NH_4)_7Mo_4O_{28}$ 0·0012; pH 5·5–6·0. Iron was given as ferric citrate usually at 0·1 m.eq./l. (1·87 p.p.m.) or 0·075 m.eq./l. (1·4 p.p.m.). In 1947 magnetite, ground to pass 100 mesh was used at a concentration of 100 p.p.m. in the sand as an alternative to ferric citrate. Control cultures were also grown without iron. In 194 $Ca(NO_3)_2$ and KNO_3 were decreased to provide a total of 8 m.eq./l. NO_3^-.

The respective 'heavy' metals were given in the nutrient solution at equivalent concentrations according to valency, and applied after plants were established.

In 1947, Cr^{+++}, Mn^{++}, Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}, Pb^{++} at 0·5 and 1· m.eq./l. and CrO_4^{--} to give the same weight of Cr as Cr^{+++} were used. In 1948 these metals were again given at 0·5 m.eq./l., including CrO_4^{--} and also Ti^{+++}, VO_3^-, AsO_4^{---}, Cd^{++}, Sn^{++}, MoO_4^{--}, Ce^{++++}, WO_4^{-}, Pb^{++}, and Tl^{++}. In 1949 the metals used in 1947 were tested at 0·2 m.eq., but Ni^{++} and Pb^{++} were omitted and Cd^{++} and MoO_4^{--} were included as in 1948.

The crops grown were sugar beet in 1947–9 and tomato ('Market King'), kale ('Marrowstem'), oat ('Star') and potato ('Majestic') in 1948.

RESULTS

a. Sugar beet, 1947–9. The behaviour of sugar beet was relatively simila from year to year where treatments were repeated, and the results are therefor presented as a whole.

Sugar-beet plants given iron either as citrate or magnetite without heav metals grew normally. Plants not given iron developed finely patterned ervenal mottling in young leaves followed by acute chlorosis and inter-al necrosis. The addition of various heavy metals caused total or intervenal orosis in young leaves which ranged from severe to trace according to the al, as indicated in Table I. Brown intervenal necrosis usually developed in orotic leaves except with Cd^{++}. There was little or no necrosis with Cr^{+++}, $^{++}$, and Pb^{++}, where chlorosis was slight. Evidence that the chlorosis was e to interference in iron metabolism was obtained in 1947 by painting leaf urfaces with 0·25 per cent. FeSO$_4$.7H$_2$O. Recovery followed after 5 days in treatments except Ni^{++} where the plants were severely injured and did not pond. Similar recovery occurred in 1948 and was observed also in plants en Cd^{++}; chlorotic plants are shown in Pl. I*d*.

ading, tinting, and necrosis distinct from that associated with iron defi-ncy were observed in older leaves of plants given Ni^{++} and Co^{++}. Necrosis nmenced as narrow brown areas close to the major veins and rapidly read into the whole area between pale green veins. Young leaves blackened l were killed. Necrosis often preceded chlorosis with Ni^{++} and then inter-ed with the estimate of chlorosis. Symptoms of manganese deficiency peared in older leaves simultaneously with iron deficiency in the young aves of plants given Zn^{++}. Cd^{++} caused almost total chlorosis of old leaves contrast to Cu^{++}, and lesions similar to those due to potassium deficiency ewitt, 1945) developed in the petioles. Most of these symptoms are recorded colour photography in a book by Wallace (1951).

. *Tomato, potato, oat, and kale* (*1948*). The relative ability of the metal s to induce chlorosis varied greatly according to the crop. The symptoms oduced did not always resemble those of true iron deficiency (Table I).

Co^{++} induced unusual symptoms in tomato and potato. The outstanding ual effect appeared to be the *increase* in chlorophyll in veins, leaf margins, d in lower leaves, which were unusually dark blue-green. In tomato expand-g leaves showed bright intervenal yellow or orange mottling with small crotic spots along major veins and midrib. These symptoms were initially ggestive of manganese deficiency (Pl. II*a*). Young leaves became malformed, g-like, and resembled those treated with certain plant growth substances; wering trusses proliferated but remained rudimentary (Pl. I*f*).

Two distinct patterns later appeared in potato (Pl. II*g*); neither was aracteristic of a known deficiency. Kale exhibited a pattern distinct from at of iron deficiency in addition to other effects such as marginal necrosis d cupping and distintegration of the lamina (Pl. I*e*).

Ni^{++} also caused unexpected effects. Chlorosis in potato was accompanied intervenal black rounded necrotic spotting in a pattern closely resembling anganese deficiency (Pl. II*d*). Black necrotic elongated areas appeared in ems and petioles. Old leaves became orange and withered, followed by ath of the growing point. In tomato yellow chlorosis between bright green ins with brown necrotic spotting developed in expanding leaves prior to pid death of the shoot, and closely resembled manganese deficiency (Pl. II*e*). lder leaves became dark green, and stems developed scaly surface lesions,

TABLE I

Summary of effects of heavy metals on various crops

Fe, or Mn signifies diagnosis of symptoms of deficiency of Fe or Mn. + + +, &c., indicate relative severity of chlorosis in entries under the left bracket. Depression of growth (shown in the last four columns) is indicated thus: +++++ growth below 10% of control; ++++ 10–30%; +++ 30–50%; ++ 50–70%; + 70–90%; – above 90%.

Metal ion supplied	Effect on chlorosis and nature of disorder diagnosed										Effect on vigour			
	Sugar beet		Tomato		Potato		Oat		Kale		Tomato	Potato	Oat	Kale
Cr^{+++}	++	– Fe	+	– Fe			–	–	–	–	+	+	+	–
CrO_4^{--}	+++	– Fe	+	– Fe	–	CrO_4 tox.	–	–	–	–	++++	++		
Mn^{++}	+	– Fe	–	Mn tox.	·	Mn tox.	–	–	+	Mn tox.	+	+		
Co^{++}	++++	– Fe Co tox.	+++	– Mn Co tox.	+++	Co tox.	++++	– Fe	+++	Co tox.	++++	+++	++++	+++
Ni^{++}	+++	– Fe Ni tox.	++	– Mn Ni tox.	++	– Mn Ni tox.	+++	Ni tox. ? – Fe	++	Ni tox.	+++++	++++	+++	++++
Cu^{++}	++++	– Fe	+++	– Fe	++	– Fe					++	++		
Zn^{++}	++	– Fe – Mn	+	– Fe			+	– Fe	+	– Fe	–	+	+	–
Ti^{+}	+													
VO_3	++	– Fe												
AsO_4	+													
Cd^{++}	++++	– Fe												
Sn^{++}	–													
MoO_4	+	– Fe												
Ce^{+++}														
WO_4														
Pb^{++}	+	– Fe												
Tl^{++}	+	Tl tox.												

ecially around leaf bases. Kale (Pl. I*c*) developed finely patterned inter-nal chlorosis followed by severe necrosis and scorching. Leaves became dimentary with bare stunted petioles. Scaly lesions formed on stems. Oat owed a characteristic *oblique* banding of alternating green and white areas l. II*f*); later tillers were totally yellow. Co^{++} and Ni^{++} caused tuber injury potato and Co^{++} caused a related effect on tomato fruit, shown in Pl. II*b* d *c*.

DISCUSSION

These experiments confirmed that several 'heavy' metals may induce symp-ms of iron deficiency more readily than manganese. Valency of the metal as sometimes a factor, but the relative oxidation-reduction potentials of the *nple* ions were not related to results, and ability to show valency change was t an essential factor in determining the incidence of an induced deficiency.

Crop susceptibility differed considerably. Sugar beet was especially sensi-ve to all metals known to induce iron deficiency except Mn^{++}, to which it owed considerable tolerance both with respect to induced chlorosis and her aspects of manganese toxicity (Hewitt, 1948*b*).

Cu^{++} consistently induced iron deficiency in susceptible crops, but some etals, e.g. Co^{++} or Ni^{++}, induced effects that were not typical of iron eficiency. Scharrer and Schropp (1934) also noted the production of a dark ue-green tint in some experiments and chlorosis in others with several reals, peas, and beans which had been given Co^{++}. Nicholas (1951) con-med certain of the symptoms induced by Co^{++} and Ni^{++} given to tomato ants in an acid soil, but the conclusion that iron deficiency was the typical sponse may require confirmation.

The appearance of apparent manganese deficiency in tomato and potato hich had been given Ni^{++}, in sugar beet with Zn^{++}, and in tomato with o^{++}, suggests that other micronutrient deficiencies may result from heavy etal excesses and that Ni^{++} and Co^{++} in particular may disturb manganese etabolism. The absence of these symptoms due to Co^{++} or Ni^{++} when given sugar beet and oat, which are normally highly susceptible to manganese eficiency, is therefore interesting.

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Thermonasty in Tulip and Crocus Flowers

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SUMMARY

The earlier literature on thermonasty is summarized, and it is confirmed that these movements are due to different growth-rates on the two sides of the perianth members.

The underlying mesophyll cells are chiefly concerned; those of the outer surface have a temperature optimum for growth about 10° C. lower than the corresponding cells of the inner surface.

The percentage elongation of complete tepals is shown to be greater than that of separate tissue strips, and this difference is shown to result from the change in internal CO_2 concentration.

Increasing the CO_2 concentration causes a marked cell extension and a lowering of the temperature optimum for growth. This effect appears to be closely related to respiration.

INTRODUCTION

THE problem of the opening of certain flowers in sunlight was first investigated during the last century. In crocus and certain varieties of tulip it was demonstrated that the movement is dependent on a rise in temperature, and is unaffected by light intensity. The change of temperature required may be small: Pfeffer (1897) records that a rise of 0·5° C. is sufficient to cause an appreciable opening in crocus, while Andrews (1929) recorded a detectable opening movement with a temperature rise of only 0·2° C. The tulip is rather less sensitive: Pfeffer (1897) recorded that a minimum temperature rise of 2° C. was required, and Andrews (1929) a rise of 1° C. Opening of the flowers both in crocus and in tulip is normally followed by a regulation ('Rückregulierung') movement in the direction of closing which adjusts the flower to its final position at that temperature.

A fall in temperature causes closing of the flower. The minimum fall required is about equal to the rise necessary to effect opening, but it is only flowers which have already opened that respond to such small changes. There is no regulation movement following closing. Böhner (1934) states that in tulip there is a critical temperature below which flowers have a tendency to close and above which they have a tendency to open. Flowers maintained below the critical temperature never open, but may grow extraordinarily in length.

Farmer (1902) suggested that these movements were due to turgor changes, but his evidence is not decisive. Other authors agree that both in tulip

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(Pfeffer, 1897; Jost, 1903; Wiedersheim, 1904; Bünning, 1929; Böhner, 1933 and 1934) and in crocus (Pfeffer, 1897; Jost, 1903; Wiedersheim, 1904) the movements are effected by the different rates of growth of the two sides of the perianth members at different temperatures. On warming, the inner surface of the tepal shows a rapid increase in length; as this occurs the outer surface shows no change in length or may even shorten. The latter is almost certainly a mechanical effect due to the sudden rapid growth of the other side (Pfeffer, 1897).

Wiedersheim (1904) investigated the effect of temperature rise on flowers of tulip and crocus artificially fixed (by wires) in the closed position. He showed that under such conditions both sides of the perianth members increased in length. Bünning (1929) demonstrated this for tulip in another way. He warmed the outer surface of the tepal while keeping the inner cool, and produced a small closing movement, thus showing that the outer surface also responds to temperature rise by growth. In general, it is agreed that the closing movement is due to the more rapid growth of the outer surface on cooling. At low temperatures the inner surface grows extremely slowly.

The regulation movement following opening was attributed by Pfeffer (1897) and Wiedersheim (1904) to the slower response of the outer surface to temperature rise, bringing about a small closing movement after the reaction of the inner surface is finished; Jost (1903) attributed the movement to 'autotropism'. Bünning (1929) suggested a different explanation for this phenomenon. He demonstrated that the outer surface reacts to temperature rise at the same rate as the inner surface and therefore rejected Wiedersheim's explanation. He suggested as an alternative that the regulation reaction was due to a mechanical stimulation of the cells of the outer surface caused by the rapid lengthening of the opposite side. He found that stroking the outer surface with a needle caused elongation of the cells in that region resulting in a closing movement. If the stimulus was provided by stretching, it had to be sufficient to cause a passive elongation of 1·5 per cent. before any response followed.

Märkert (1931) investigated the effect on the position of the tepal of removing either one or both epidermes, and concluded that the growth reaction occurs chiefly in the cells of the mesophyll. Böhner (1933, 1934) investigated the same problem by measuring changes in length of the two epidermes on removal from closed and open flowers. He concluded similarly that the chief mechanism is a change in length of the mesophyll cells controlled partially by the epidermes which are under some tension. Märkert also carried out a number of potometer experiments and showed that there was an increase in water uptake during both opening and closing. She suggested that this extra water absorption is due to an increase in the osmotic pressure of the mesophyll cells. However, Bünning (1929) measured the osmotic pressure (using a plasmolytic method) and recorded a decrease in the value of some 0·02M. for the cells of the inner surface after warming.

Changes in elasticity and plasticity were measured by Bünning (1929) and

Böhner (1934). Böhner concluded that there was little change in either elasticity or plasticity on warming. He suggested that the increase in length is due to a conversion of the elasticity already present into plasticity, this being effected by water uptake.

Bünning showed that when cells were poisoned by ammonia vapour no response occurred. Living cells are therefore involved.

Wiedersheim investigated the behaviour of crocus flowers rotated on a clinostat and found that they responded quite normally to temperature changes.

A number of interesting points emerge from this earlier work. The movements appear to be due to true growth reactions, and not to reversible turgor changes, as there is little change in osmotic pressure, and a permanent increase in length follows every reaction. The two surfaces of a tepal obviously have different temperature optima for growth.

Calculations were made from the data of Pfeffer, Jost, and Wiedersheim of the rates of growth at different temperatures in the steady state (flower neither opening nor closing). The temperature coefficients (Q_{10}) so obtained had values between 2 and 5 over a temperature range from 5·5° C. to 26° C.

During opening the temperature coefficients reach unusually high values—between 20 and 30—for the cells of the inner surface. The reverse occurs during closing when the cells of the outer surface have Q_{10} between 0·1 and 0·2, owing to the fact that they are growing more rapidly at a low temperature.

In the experiments of Wiedersheim (1904) in which growth rates were measured on fixed perianth members, the temperature coefficients are normal, showing that the extra reaction has been prevented.

A typical effect of temperature on growth is therefore insufficient to explain these thermonastic movements.

EXPERIMENTAL METHODS

Pink tulips (variety 'William Pitt') were used in all experiments unless otherwise stated. Three types of measurements were used.

1. *Angle of opening*. This was measured on a single intact perianth member left attached to the flower stalk. The shape of the tepal in the median line was traced, and the angle measurement made by taking the tangents to the tip and to the base of the tepal as in the pea test for auxin. This was used only as a rough measurement.

2. *Measurement of growth on complete flowers*. All the tepals except one were removed and both the inner and outer surfaces of this marked in the median line with spots of Indian ink, about 2 to 3 mm. apart. The distances between these were measured with a travelling microscope. Errors due to the curved surface of the tepal were reduced by having the spots close together.

3. *Elongation of strips*. Strips of tissue were obtained by splitting the median portion of the tepal into two halves longitudinally (one consisting of outer cells, mesophyll and epidermis, and the other of inner cells, mesophyll and epidermis). These strips were marked with two spots of Indian ink about

1·5 cm. apart in the rapidly-growing region, clamped between the two halves of a cork, and suspended in water in a battery jar. To prevent curling, a 20-g. weight was suspended with cotton from the lower end. This caused very slight stretching, but did not affect the normal response of the tissue. The battery jar was surrounded by a water-jacket in which the temperature could be rapidly changed. The distance between the marks was measured at intervals with a travelling microscope.

RESULTS

I. *Growth measurements*

(*a*) *The behaviour of the two surfaces in complete perianth members.* Using the experimental method described previously, the behaviour on warming of the two surfaces of the tepal was investigated in *Crocus vernus* and tulip. The results confirm those recorded in the literature. The inner surface grew very rapidly at first and after about 45 minutes slowed down to a fairly constant rate. The outer surface stopped growing or even decreased in length (crocus) during this time. On cooling, the outer surface grew rapidly, whilst the inner grew extremely slowly.

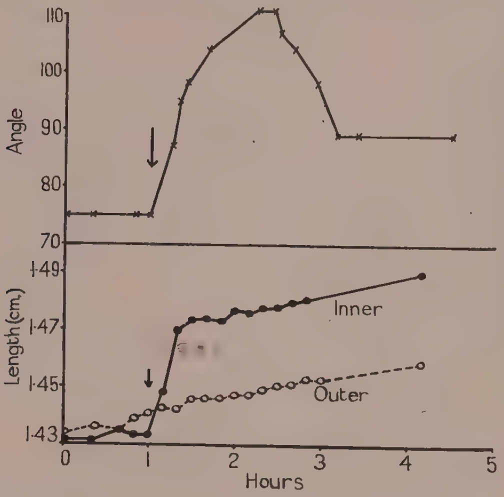

FIG. 1. The effect on a complete tepal (upper half) and on strips of inner and outer cells (lower half) of raising the temperature from 7° C. to 17° C. at ↓.

It was decided in all future experiments to investigate the behaviour of the two surfaces separately.

(*b*) *The effects of temperature change on separate strips.* Strips were obtained as previously described, and their typical behaviour on warming is shown in Fig. 1. For comparison, the angle of opening of a complete flower was measured under identical circumstances. A typical opening occurs followed y the ‘back regulation’ adjusting the flower to its final position at that emperature.

In separate strips no decrease in length of the outer surface was ever found. t was therefore assumed that this is dependent on the rapid growth of the nner surface, and that the ‘back regulation’ results from the recovery from ension as the rate of growth of the inner surface slows down.

In the equilibrium position the two surfaces grow at approximately the same ate.

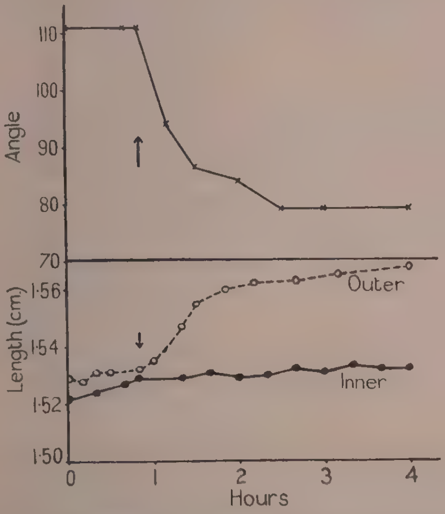

FIG. 2. The effect on a complete tepal and on strips of inner and outer cells of lowering the temperature from 20° C. to 10° C. at ↑. Conventions as in Fig. 1.

On cooling flowers which have reached equilibrium the cells of the outer surface grow rapidly at first and later more slowly (Fig. 2). The cells of the inner surface grow very slowly at lower temperatures. No ‘back regulation’ follows closing.

A curious feature was noticed during these experiments which was that a much greater growth reaction is found in complete tepals than in separate strips (Table I) during both opening and closing of the flower.

TABLE I

Percentage increase in length of inner surface of tepal on warming from 7° C. to 17° C.

Complete tepal	Strip of inner surface
+5·1%	+2·6%
+7·3%	+1·8%
+6·1%	+1·0%
+5·8%	+2·2%

(*c*) *The cells concerned in the movements.* The epidermes were stripped from a petal and their behaviour investigated separately, using the same technique as for isolated strips. The outer epidermis responded to a low temperature by more rapid growth, while the inner showed faster growth at a higher temperature.

The reactions, although similar in kind, proved to be of smaller magnitude than those of the corresponding mesophyll cells, and it is therefore concluded that it is the mesophyll cells which play the major part. These cells are elongated and have thin walls, a structure such as to facilitate great increase in length on water uptake.

II. *Osmotic relations*

The osmotic pressure of the cells of the inner epidermis was investigated before and during warming by the 50 per cent. plasmolysis method (in sucrose and in KCl) and a change was found from an initial value of 8·4 atmospheres (at 7·5° C.) to 8·2 atmospheres after 1 hour at 18° C.

The mesophyll cells were more difficult to investigate as they are colourless, but previous staining in dilute neutral red allowed measurements to be made. On warming, the osmotic pressure of the inner mesophyll cells showed a change from 7·5 atmospheres at 4° C. to 7·3 atmospheres after 30 minutes at 15° C. The outer epidermis and mesophyll also showed a very light decrease in O.P. on cooling. Such small changes are within the experimental error.

In crocus the osmotic pressure was determined cryoscopically. At 5° C. the O.P. of a whole tepal was 10·2 atmospheres, while after 1 hour at 15° C. the osmotic pressure was 9·1 atmospheres. The large decrease here is presumably due to the rapid water uptake which accompanies opening of the flower. It was noticed during these investigations that the cells plasmolyse rapidly both at high and low temperatures. It therefore appeared unlikely that permeability changes have a significant effect.

The change in pH was measured on warming from 5° C. to 18° C. for 30 minutes. Initially the value was 5·62 and finally 5·64.

The reducing sugar content was measured according to the method of Van der Plank (1936) and the 'glucose' varied from an initial 4·13 mg./tepal to a final 3·32. In crocus at 5° C. the 'glucose' was 15·20 mg./tepal and on warming to 18° C. for 1 hour this became 15·22 mg./tepal. The titratable acidity varied from 0·024 mg. eq./tepal to 0·026 mg.eq. after warming.

It is concluded from these results that neither changes in osmotic pressure of the cell contents nor changes in permeability are sufficient to explain the phenomenon.

III. *Metabolic changes*

(*a*) *Respiration rates.* Respiration is known to be intimately associated both with water uptake (Kelly, 1947) and with growth, and therefore oxygen intake and carbon dioxide output were investigated using the Warburg direct method.

Fig. 3 shows the typical respiration of tepals kept at a constant temperature f 14° C. until equilibrium was reached, and then rapidly heated to 25° C. There is a sudden increase both in oxygen intake and in carbon dioxide output which is associated with the phase of rapid elongation of the inner mesophyll cells. This is followed by a fall to a steady rate associated with the similar steady rate of growth.

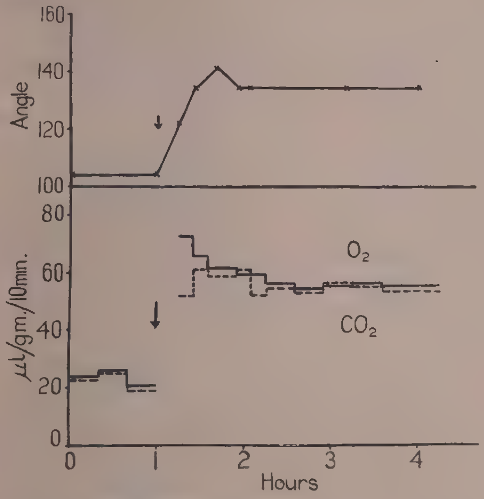

FIG. 3. The respiratory behaviour of pieces of tepal on warming from 14° C. to 25° C. at ↓, compared with the opening reaction in a whole tepal on a similar temperature-change (shown in upper half of figure).

Tepals heated from 9° C. to 19° C. behaved in exactly the same way.

The respiration of separate halves of the tepals was investigated, and it was found that the inner surface has a higher rate of respiration (—82 μl./g./hr. O_2 intake) than the outer (—54 μl./g./hr.). The behaviour on warming is slightly different; the inner surface behaves similarly to the whole tepal, while the outer shows no sudden increase but a steady rise to the equilibrium rate. The temperature coefficients (Q_{10}) at equilibrium are identical at 2·7.

Fig. 4 shows the effect on respiration of cooling the perianth from 20° C. to 9·5° C. A falling rate of oxygen intake accompanies closing of the flower. This experiment was repeated on a different variety of tulip which showed no characteristic closing movement. The behaviour was similar, although a continuously lower rate of respiration was found.

Table II shows the respiration for tepals in equilibrium at any one temperature. The temperature coefficients (Q_{10}) have values between 2 and 4 and are therefore similar to those recorded for growth. The extremely high temperature coefficients for growth recorded immediately after warming are associated with the initial high rate of respiration; but the temperature coefficient for this stage is still considerably lower (5–10) than that for growth (20–30).

(*b*) *Effects of different treatments on respiration and growth.* Treatments calculated to affect the rate of respiration were tried. Under anaerobic conditions (nitrogen atmosphere) no typical thermonastic movements occurred in either tulip or crocus. Sodium azide (10^{-3}M.) prevented all perceptible growth and thermonastic reaction. These results are similar to those of Kelly (1947) for oat coleoptiles. She found in that tissue a close relation between respiration and water uptake.

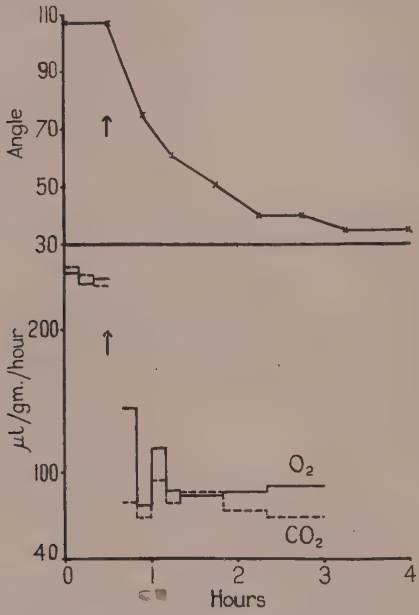

FIG. 4. The respiratory behaviour of pieces of tepal on cooling from 20° C. to 9·5° C. at ↑, compared with the closing reaction in a whole tepal on a similar temperature change. Conventions as in Fig. 3.

TABLE II

Respiration rates of whole tepals

Temp.	O_2 intake µl./g./hr.	CO_2 output µl./g./hr.	R.Q.
9°	−104	+86	0·83
14°	−144	+135	0·94
19°	−294	+282	0·96
25°	−336	+312	0·93

Temp. coefficients 9°–19°: O_2, 2·83; CO_2, 3·28; 14°–25°: O_2, 2·34; CO_2, 2·50.

Sodium citrate (0·01 M.) promoted respiration by some 28 per cent. at 14° C. and by 50 per cent. at 25° C. and also increased the growth of the inner cells to a lesser extent, giving an increase in growth of 3·34 per cent. for a temperature rise from 10° C. to 14° C. compared with only 2·90 per cent. in water.

(*c*) *The effect of carbon dioxide.* It has been shown by Mer and Richards 1950) and Mer (1952) that carbon-dioxide concentration markedly affects the growth of the oat mesocotyl. Different concentrations of carbon dioxide were therefore tested on tulips.

At a low temperature (8° C.) 10 per cent. CO_2/air mixture promoted a further closing movement. At a higher temperature (16° C.) 10 per cent. CO_2/air caused a most marked opening reaction. In both types of experiment flowers were allowed to reach equilibrium at the temperature concerned before treatment.

The method involved enclosing the flowers in litre flasks through which the gas mixture was passed. The possibility remained that humidity changes might therefore be affecting the response. This factor is obviated in the next series of experiments in which the effect of CO_2 concentration was tested on strips immersed in water. For every temperature the perianth members were allowed to reach an equilibrium before being tested. The inner and outer surfaces were investigated separately. The rates of growth are calculated as percentage increases on the original length during the first hour of response.

At 8° C. with either CO_2-free air or a rapid stream of air bubbling through the water both the inner and outer surfaces grow very slowly. Increasing the CO_2 concentration causes a steady increase in the rate of growth of the outer cells. The cells of the inner surface behave very differently. Extra CO_2 causes no increase in length until a value of over 2 per cent. CO_2 is reached. In 10 per cent. CO_2 they grow more rapidly than the outer cells (Fig. 5).

At 15° C. the effects are different. At low CO_2 concentrations both surfaces grow slowly (the outer faster than the inner). As the CO_2 concentration increases, the rate of growth of the outer surface decreases somewhat, while the inner surface shows an increase, this being greatest at high CO_2 concentrations (Fig. 5).

Similar results are shown in Fig. 6. In air the cells of the inner surface have a temperature optimum for growth between 17° and 25° C. The corresponding value for the outer surface is between 8° and 15° C. Carbon dioxide has a marked effect on this optimum, which is most clearly seen in the inner surface cells. A rise in CO_2 concentration shifts the optimum to a lower temperature. For example, in 5 per cent. CO_2 the optimum is at about 11° C. as compared with a value of over 17° C. in air. At 10 per cent. CO_2 the optimum is presumably even lower.

It seems likely that the cells of the outer surface show a similar effect which is not obvious as they already exhibit a low temperature optimum for growth.

(*d*) *Experiments on the fixation of* CO_2. Measurements were made of the rates of respiration in the presence of CO_2, using the Dickens and Simer method second (Dixon, 1934) and a Haldane gas analysis technique.

At 5° C. using the latter method, no fixation of CO_2 was found but rather

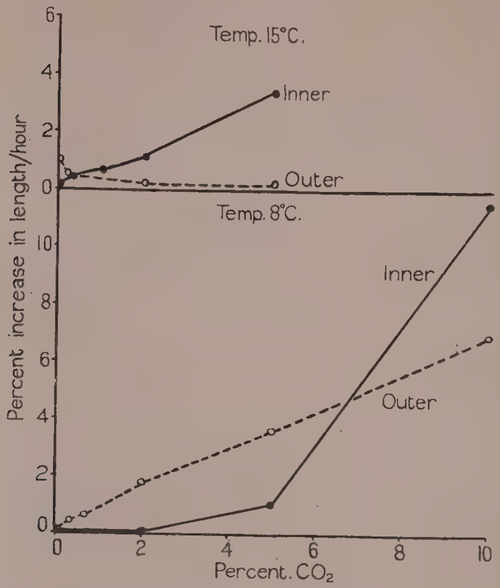

FIG. 5. The effect of different concentrations of CO_2 in air on strips of inner and outer tissue at different temperatures.

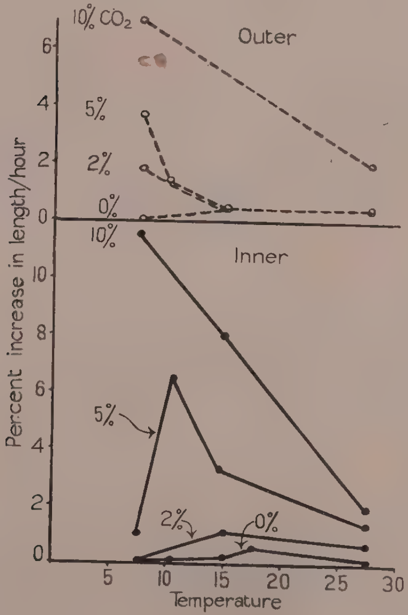

FIG. 6. Relationship between CO_2 concentration and temperature in the growth of tepal strips.

an increased output during the first 2 hours after treatment with 10 per cent. CO_2 (400 µl./g./hr. compared with an air control of 75 µl./g./hr.).

The Dickens and Simer method gave similar results, again indicating no CO_2 fixation. At 10° C. in 5 per cent. CO_2 the respiration rate was -809 µl.O_2/g./hr. compared with the value in air of -104 µl.O_2/g./hr. already given.

These experiments indicate that results obtained by the Warburg direct method in which respiration is measured in the absence of CO_2 must be interpreted with caution.

DISCUSSION

It has been shown in the previous section that in perianth members as in other elongating organs there is a correlation between the respiratory behaviour and growth. Inhibition of aerobic respiration (by anaerobic conditions or sodium azide treatment) prevents both opening and closing movements. Sodium citrate (a respiration promoter) increases growth and respiration to a similar extent.

This correlation is particularly marked during the opening movement which follows heating, but is less obvious during closing, when increased growth of the outer surface cells is accompanied by a falling rate of respiration. But during the rapid-growth phase of this surface the respiration rate is still considerably above the equilibrium value for the lowered temperature. It has already been pointed out that caution is required in interpreting these respiration data in view of the fact that CO_2 appears to have a marked effect on respiration.

Experiments on the growth of the two surfaces show that such tissues maintained in a current of air or CO_2-free air do not grow at the same rates as in a complete flower. The factor altered by this treatment appears to be the CO_2 concentration. If the separated surfaces are supplied with between 2 per cent. and 5 per cent. CO_2 in air, they react as in whole perianth members.

Measurements of the growth-rate for the first hour after treatment with 2 per cent. CO_2 gave the following temperature coefficients: for the inner cells a rise from 8° C. to 15° C. gave a Q_7 of 11, for the outer cells under similar conditions the Q_7 was 0·1. These values correspond well with those calculated for whole flowers during opening and closing.

The close correlation between the effect of CO_2 on whole flowers and on slices is shown in Fig. 7, which illustrates the reaction on passing 5 per cent. CO_2 through tissue strips at two different temperatures. Growth is rapid at first but soon diminishes, a very similar curve being obtained to that on change of temperature. Five per cent. CO_2 has no effect on the opening of whole flowers.

If it is supposed that the tepals have an internal CO_2 concentration of between 2 and 5 per cent., the growth reactions on change of temperature can be explained. Such high internal concentrations have been found for example in bogbean leaves; in darkness values between 1 and 3 per cent. have been recorded (Vallance and Coult, 1951) and much higher values, 10 per cent. CO_2 or even greater, are known in the more bulky apple tissue (Hulme, 1951). Rough estimates of this quantity in tulip were made by determining the bound CO_2, finding the volume of air space by weighing before and after injection with water, and hence calculating the required percentage. This indicated an approximate value of 5 per cent.

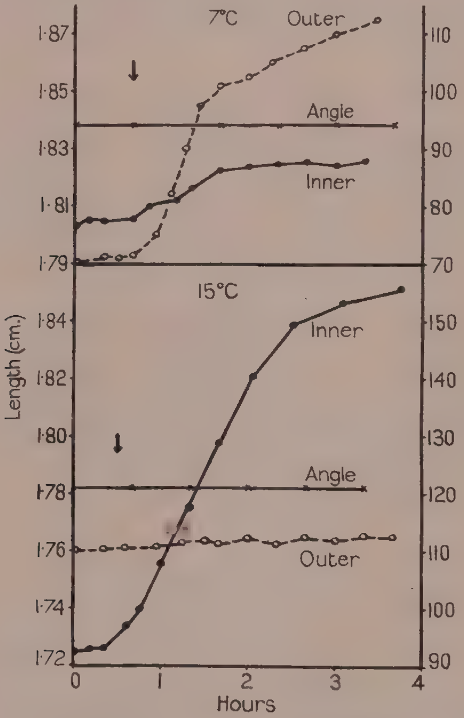

FIG. 7. Effect of 5% CO_2 on the angle attained by whole tepals, and on length of tissue strips at two different temperatures.

It is considered therefore that at least two factors are concerned in thermonastic movements. Firstly, the existence of two adjacent tissues having very different temperature optima for growth. The cause of this is unknown and may be connected with different amounts of growth substance or with different efficiencies in the coupling of respiration and growth. Secondly, the tissue appears to have a high internal CO_2 concentration, the response to which on temperature change gives the rapid rates of growth observed. It is this factor which is responsible for the difference in behaviour of whole tepals and isolated tissue strips.

The action of carbon dioxide on elongating cells is a phenomenon of some interest which is not confined to this tissue (Mer, 1952; Harrison, 1952). A urther study of this problem may help in the elucidation of the relationship etween growth and respiration. An indication is given from the present nvestigation that an increased rate of respiration is produced by enriching he atmosphere with carbon dioxide.

My thanks are due to Professor W. T. Williams for his stimulating interest n this work and helpful criticism of the script.

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The Distribution of P^{32} in the Plum and its Mutagenic Possibilities

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SUMMARY

The occurrence and utility of bud sports in fruit-trees are discussed and the merits of various mutagenic agents compared. P^{32} is suggested as a suitable mutagen in fruit-tree mutation studies and experiments are described on the uptake and distribution of P^{32} in plums. The uptake in ten 3-year-old trees cultured in sand appeared to be of the order of 10 to 30 per cent. increasing with the vigour of the tree. The P^{32} was distributed mainly in the shoots, the average initial concentration being 1·5 μc./g., and in their buds, average initial concentration 3 μc./g. The latter figure corresponds to a dose to the buds of the order of 2,000 r., and although this is a dosage level at which mutagenic effects are likely to occur, there was little sign of macroscopic damage. The method therefore appears to be a suitable one for fruit-trees.

INTRODUCTION

THE relative merits of a number of mutagens for use in the production of 'bud sports' in fruit-trees are considered in this paper. In particular an alternative is sought to X-rays, which are unsuited to field work and produce gross morphological damage.

Vegetative 'sports' or somatic variations are a feature of clonally propagated cultivated plants. They are of value in the production of new varieties in that they change a particular character or groups of characters without disturbing the rest of the genome. However, like all mutations, the majority of variants are a disadvantage to the clone and, where such variants are difficult to detect, they constitute a menace to the purity and productivity of a clone. A number of deleterious changes are common in potatoes, for instance (McIntosh, 1945). Mutations which would be deleterious to the plant as an evolving species may sometimes be economically advantageous. Such paradoxical situations occur in the breakdown of self-incompatibility mechanisms and in the mutation to male-sterility (Lewis, 1946). The practical value of mutations in agricultural plants in general has been reviewed by Gustaffson (1947).

The frequency and variety of sports in apples is shown by the survey of Shamel *et al.* (1933) and also by Granhall (1950). A general review of bud mutations is given by Shamel and Pomeroy (1936). Bud sports are often chimerical in structure (Marks, 1952); that is, only one of the histogen layers which delineate the shoot possess the altered constitution. However, as bud

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ports are, in the main, used as individuals rather than as suitable parents, his structural feature need not affect their utility.

The production of somatic mutations. The principles governing the induction f mutation in the germinal and in somatic tissue are similar, but the techiques used to transfer the necessary amount of energy either in a labile molecule or through irradiation vary considerably.

Three methods of effecting this energy transfer are possible. First, by introducing a molecule to the appropriate point. In this method accurate control of the mutagenic agent is possible, but the types of molecule used diffuse with difficulty to the point of action. In somatic mutagenesis the subject-issue is often surrounded by layers of differentiated tissue and the effective dose is only an incalculable fraction of the total dose given. This problem and the allied one of toxic effects in the surrounding tissue are discussed by Auerbach (1949).

In the second method mutation is brought about by some external source of radiation such as X-rays, ultra-violet light, neutrons, or alpha-particles. Relatively accurate dosage control is possible, but the undesirable irradiation of 'supporting tissue' is difficult to avoid.

Thirdly, there are methods involving the use of radioactive isotopes. These sotopes can be employed as external radiation sources, e.g. radon and Co^{60} (Lorenz, 1950), or as systemic mutagens. In the latter case the efficiency of action of an isotope varies according to the manner in which it is selectively concentrated in tissue and depends on the half-life and mean energy of the emitted radiation. Systemic mutagens of this type (P^{32}, S^{35}, and Na^{22}) are compared one with another and with X-rays and neutrons by Gustaffson *et al.* (1950). These authors show that the advantages of the selective concentration of P^{32} and its convenient half-life outweigh the low ion density due to the high energy of its beta-particles.

P^{32} has been shown to be a satisfactory mutagen for a variety of subjects. Bateman and Sinclair (1950) show that it is comparable with X-rays for the production of visible and lethal mutations in *Drosophila*, while Rubin (1950) has shown that it may be more effective than X-rays for the production of mutations in bacteria, for the same dose. Other reports suggesting that P^{32} is at least as efficient a mutagen as X-rays have recently been published.

The results of X-irradiation and of treatment with nitrogen mustards and P^{32} of apples and pears have been described by Granhall *et al.* (1949). In this work excised scions were treated, but a method in which the mutagen is brought in close contact with its subject, viz. the actively dividing region of the growing point would be desirable. For this reason the use of systemic mutagens is indicated, and of these, the isotope P^{32} appears most suitable. The main problem is that of giving the buds a controlled dose. The variability of dose and its lethal effect have been considered for the potato by Stanton and Sinclair (1951). Gustaffson and Nybom (1950) showed that more active barley seeds preferentially absorbed P^{32}, and it is likely that this phenomenon of preferential demand by the stronger growing-points will occur also in trees. It may be difficult therefore to specify the amount of P^{32} to be fed to individual trees in order to provide a concentration in the shoots likely to induce mutations.

In order to extend the study of the effects of high concentration of P^{32} on growth of the potato to fruit-trees, plums were chosen as the experimental material because they are genetically simpler than the pome fruits and were considered more likely to show the effects of treatment.

The essential points appear to be: (1) the gross uptake of P^{32} by the tree for a particular method of feeding and amount administered; (2) the distribution of P^{32} in the tree, particularly the ratio of P^{32} in the growing points to the average level in the shoots; (3) the dosage level at which mutations are produced.

EXPERIMENTAL

A number of different plums were available for this work and a group was selected which represented the variation in cultivated varieties. They are listed in Table I. The trees were lifted, and after their roots were washed they were planted in 6·5-litre metal cans in acid-washed silver sand. The cans were fitted with a glass drainage tube protected by glass wool and were painted internally with two coats of a bitumen emulsion. The moisture capacity of the cans plus sand was 1·5 litres. Bottles were provided to collect the eluate.

TABLE I

Weights (g.) of plum-trees used (serial numbers of trees in parentheses)

	Stock		
	Mariana	Mussel	Myrobalan B
Bastard Victoria	—	(1) 606	—
Belgian Purple	(2) 241	—	—
Early Greengage	(3) 355	(4) 634	—
Goldfinch	(5) 294	—	—
Langley Bullace	—	(6) 335	—
Late Orange	—	—	(7) 340
Old Greengage	(8) 354	—	(9) 605
White Magnum Bonum	—	(10) 411	—

Average height, 170 cm. The laterals had been removed for the first 70–100 cm. from ground-level. Trees budded 1948 and lifted March 1951

Twice weekly the trees were given a complete major element nutrient solution in which the phosphate level was low (K 135, Ca 134, Mg 40, Fe 14, P 40, N 40 p.p.m.). A minor element solution (Mn 0·3, Cu 0·1, Zn 0·1, B 0·5 p.p.m.) was given once a week. At other times rain-water was given as required. The P^{32} was given separately on 4 May, 24 hours after the cans had been thoroughly leached with rain-water. Feeding was continued as before, but all leachings were returned to the cans for the first fortnight after treatment. At the end of this time the cans were again leached thoroughly and no further eluate returned. The trees showed up to 15 cm. of extension growth at the time of treatment. 1·5 mc. of P^{32} was given to each tree in 200 ml. of 0·002 M. Na_2HPO_4. With a full recovery of this in the tree, it would allow a mean specific activity of 3 μc./g. fresh weight at the time of application.

Measurement of P^{32} activity. One week after treatment the surface of the rees was surveyed for activity using a GM-4 end-window counter. A limiting iaphragm of brass was used over the counter window so that the beta-articles of P^{32} were admitted through a circular aperture 8 mm. in diameter. Because of the short range of the beta-particles, the results of this survey would be a measure of the concentration of P^{32} in the surface layers of tissue mmediately below the counter aperture.

In order to provide more specific information on the average concentration f P^{32} throughout the whole plant tissues, samples were taken from each tree s follows. One shoot was taken from near the apex of each tree and another called the 'mid-stem' shoot) from a point about 50 cm. lower down the main tem. The shoots were cut to the base of the current year's wood and sub-livided into (1) stem+lateral buds, (2) leaves and petioles, (3) apical bud nd enfolding leaves. These 6 samples from each tree were weighed fresh, lried, crushed, and wet-digested. The digested samples were measured in a iquid counter and the total activity of each was estimated from the counter alibration which is based on absolute beta-counting.

RESULTS

Surface survey. The results of the survey over the surface of the trees are given in Table II. The trees have been placed in order of increasing weight i.e. in order of increasing vigour) to show the effect of this variable on the other quantities. The results given in the table for buds are the averages of measurements on about 25 buds per tree. As the results for individual buds varied considerably, the standard deviation has been included. It is evident that there is a tendency for the average bud activity to increase with tree weight. The low and relatively constant values of the surface counting rate for the stems suggests that comparatively little of the P^{32} is retained at least in their surface layers. The activity of the young leaves appears to be about

TABLE II

Counting rates, in counts per second, measured at the surface of the tree tissues 7 days after treatment

		Activity-counts per second				
Tree No.	Initial weight g.	Bud Mean±σ	Stem (Approximate values)	Leaf (Approximate values)	Sand	Eluate
10	241	85±26	20·0	70	200	20
2	294	62±38	5·5	20	720	25
4	335	58±46	7·0	25	360	25
1	340	166±108	17·0	70	220	22
8	354	27±21	7·5	15	620	35
6	355	107±53	15·0	35	320	22
9	411	185±137	14·4	35	320	15
3	605	111±41	15·8	40	250	5
7	606	198±107	12·8	50	250	50
5	634	71±15	15·0	30	320	18

half that of the buds. The high values of counting rates over the sand surface is due to a high localized adsorption of P^{32} in the surface layers, and these values appear to decrease with increasing tree weight.

Digested samples. The results of the counting on digested samples are expressed in μc./g. fresh weight as this is a useful quantity from which the tissue dose may be estimated (Marinelli, 1942; Mayneord, 1950; Stanton and Sinclair, 1951).

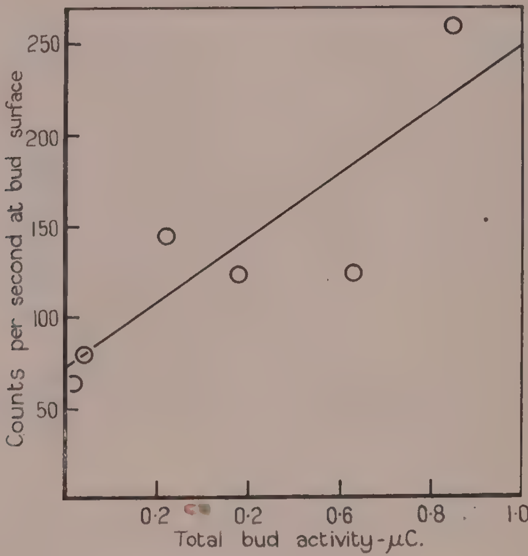

FIG. 1. Counting rate at the surface compared with bud activity.

In order to compare the surface measurements with the digested sample results for apical buds it is more convenient to express the latter in terms of total activity in μc. The surface measurements on buds are more nearly indicative of this quantity since the buds were small compared with both the beta range and the counter aperture. The results of both measurements for six buds are shown in Fig. 1, from which it is evident that the quantities are approximately proportional to one another.

The difference between the average activity of the terminal shoots and that of the mid-stem shoots is shown in Fig. 2. The points plotted for each tree lie close to the 'equivalent activity' line with the exception of points 7 and 9. The latter two points refer to trees grown on Myrobalan B root-stock, and it appears possible that this vigorous root-stock affects the distribution of P^{32}. The closeness of the points for the other trees to the equivalent activity line suggests that the two shoots per tree may be representative of all the shoots on that tree. In subsequent graphs the terminal and mid-stem shoots are plotted separately, but the differences between them are evidently small.

As the weight of a shoot is a measure of its vigour, the mean activity of the

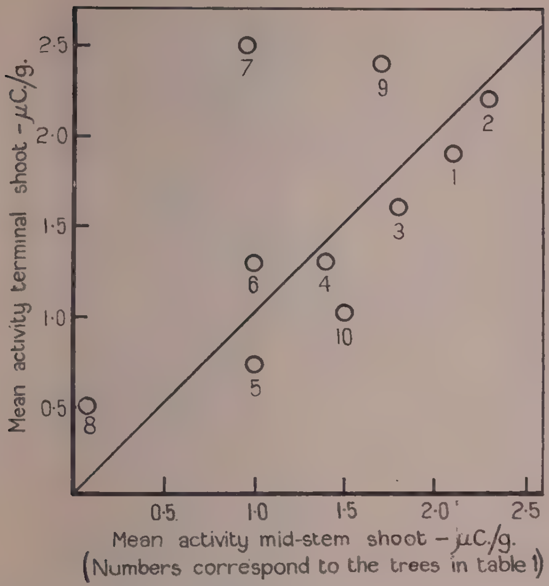

FIG. 2. Correlation between terminal and mid-stem shoot activity on the same tree.

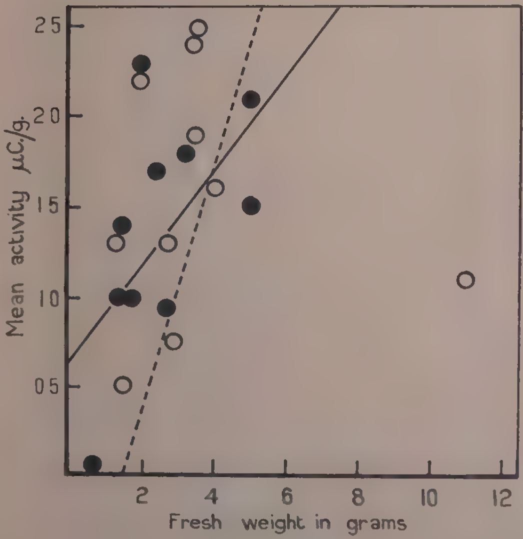

FIG. 3. Activity of the shoots compared with their weight. Mid-stem shoots —●—; terminal shoots – – ○ – –.

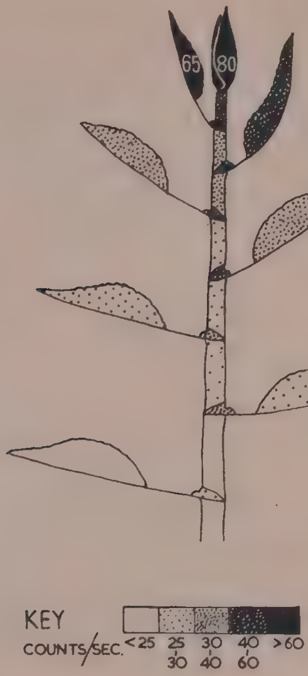

FIG. 4. Distribution of counting rate over the surface of a shoot

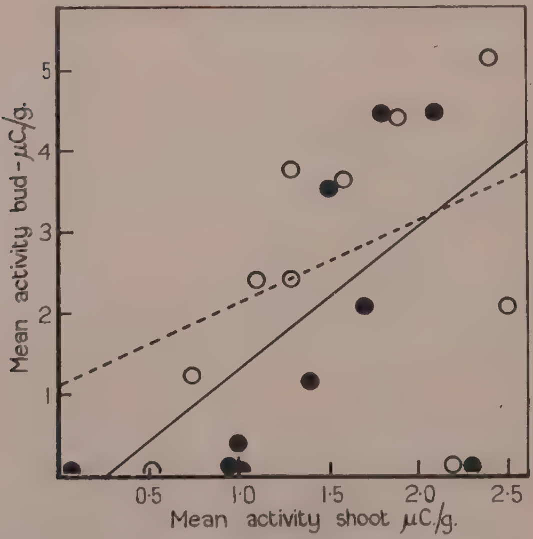

FIG. 5. Terminal bud activity compared with its shoot activity. Mid-stem shoots —●—; terminal shoots – – ○ – –.

hoots is plotted against their weight in Fig. 3. The steepness of the slope of he lines shows the importance of the effect of vigour on the distribution of ^{32}, and it would appear therefore that the main problem in producing uniorm activity in a population of shoots would resolve itself into equalizing heir vigour. Careful selection, husbandry, and pruning of trees is therefore ecessary in order to produce a uniform concentration of P^{32} in the shoots om different trees.

The results of measurements over the surface of a shoot are shown in ig. 4. Although there is a considerable 'activity gradient', the average conentration of P^{32} in the meristematic regions of the shoot appears to be of the rder of two or three times the average level in the whole shoot. A more exact stimate of this differential ratio is obtainable from Fig. 5, in which the mean ctivity of the buds is plotted against the mean activity of the whole shoot or the digested samples. The average ratio between the two quantities is : 1, the greatest departures from the average being when the activity is low, specially in the 'mid-stem' shoots.

DISCUSSION

The results obtained by surface counting (Table II) and from the measurements on digested samples suggest that the uptake of P^{32} increases with reater tree weight. Preliminary trials with a stem injection method of feeding, hich has the advantage of extreme practicability in field experiments, indiated that more of the administered P^{32} might be utilized than in the method sed in this work.

The general distribution of P^{32} in the plums confirmed the expectation that he highest concentration appeared in the growing regions of the tree and articularly in the buds. The mean ratio of 2 : 1 between the mean activity of he buds and that of the whole shoot was lower than that found in the potato Stanton and Sinclair, 1951).

The mean activity level of the shoots (Fig. 5) is of the order of 1·5 μc./g., hich corresponds to a total dose of about 1,200 röntgens (Stanton and Sinlair, 1951), while the mean bud activity is about 3 μc./g., equivalent to a dose f about 2,400 r. The actual dose will be less because the bud dimensions are maller than the beta range and the doses to individual buds probably range etween 1,000 and 3,000 r. with an average of about 2,000 r.

An important point in comparing the effects of P^{32} with X-irradiation is the uestion of gross morphological damage to other parts of the tree in delivering a dose of this order to the meristematic regions. With X-rays, gross morphological damage, which appears in the form of malformed leaves, bifurated shoots, twin terminal buds, and fasciated shoots, results from doses of etween 1,000 r. and 5,000 r. The visible damage observed in these experiments was splitting of terminal buds (rarely) and distorted leaves with chlorotic atches. These leaves were initials infolded around the bud at the time of rradiation and clearly received a higher dose than other leaves on the shoot. As the phenomenon of the splitting of the terminal parts of the shoot is at least partly a varietal effect, it is difficult to assess, in the small number of treatment units, how much of it was due to irradiation. In comparing P^{32} with X-rays it must be remembered that the equivalent doses in röntgens are of short duration and high intensity with X-rays, and of long duration and low intensity with P^{32}. Nevertheless this small amount of gross damage with dosage levels due to P^{32} which appear likely, from past experience, to produce mutagenic effects, is an advantage in favouring the survival of tissues derived from mutant cells.

A useful technical point arises from the fact that the results of the counting of digested samples confirm the general information obtained from the surface survey. This suggests that a surface survey may, with some refinement, be used as an indication of the dosage to the meristematic regions of tissue whose destruction for activity assay is to be avoided.

In obtaining somatic mutations of economic value in clonal plants, the handling of large quantities of experimental material is difficult to avoid. It has been shown by the observations described in this paper that P^{32} is a useful agent for the mutagenic treatment of such material and has considerable advantages over the other mutagens.

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The Development of the Banana Fruit

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SUMMARY

1. The banana fruit is either seeded or vegetatively parthenocarpic; the latter may or may not be seed fertile, depending on a complex of cytogenetical factors. Edible pulp (a starchy parenchyma), which fills the fruit in parthenocarpic types and surrounds the seeds in seeded bananas, mostly originates from the outer lining of the loculus (the innermost layer of the pericarp).

2. The graphs of growth in volume of seeded banana fruits are sigmoid in shape. Those of parthenocarpic fruits are variable but are not sigmoid and the shapes are related to specific origins.

3. Growth rates are related to certain ovule behaviours, to seed content of the fruit, and to ploidy.

4. α-Naphthylacetic acid induces parthenocarpy in seeded bananas and stimulates it in weakly parthenocarpic types. By contrast, coumarin, a hormone inhibitor, inhibits it in strongly parthenocarpic forms.

5. Physiological and genetical implications of the results are discussed. Fruit development is thought to be under hormone control and two phases of development of the parthenocarpic fruit can be distinguished. The desirability of distinguishing between the terms 'seedless' and 'parthenocarpic' is pointed out.

INTRODUCTION

EDIBLE bananas are vegetatively parthenocarpic; seedless fruits develop without the stimulus of pollination. The wild ancestors of the edible bananas, by contrast, are fully seeded; their fruits only develop if pollinated and then contain numerous hard black or brown seeds 3 to 5 mm. in diameter. Recent cytogenetical researches have shown that parthenocarpy in certain diploid families is due to a minimum of three dominant genes (Simmonds, 1953), and that the seed sterility normally associated with it is not absolute but is conditional upon a complex of cytogenetical factors such as ploidy, hybridity, chromosome behaviour, sterility genes, and so forth (Dodds and Simmonds, 1948 *a*, *b*). The studies reported here have been undertaken partly because of the intrinsic interest of the subject and partly to provide background for cytogenetical researches on *Musa*. Thus the approach is essentially cytogenetical and the results are complementary to the metabolic investigations made at this Institution some years ago (Wardlaw, Leonard and Barnell, 1939 *a*, *b*; Barnell, 1940).

Thanks are due to Dr. H. Evans and Dr. D. J. Finney for helpful advice; and to Mr. F. Pena, who made many of the thousands of measurements upon which this work is based.

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MATERIALS AND METHODS

Plant materials were the collections of wild and cultivated bananas maintained at this Institution and also various plants of experimental origin. Attention has been confined to *Musa acuminata* and *M. balbisiana* and their edible derivatives[1] (Dodds and Simmonds, 1948*a*). The edible diploids and triploids used were mostly established cultivars; the edible tetraploids were raised as hybrids of triploids with diploids. Some 50 or 60 clones have been used in one way or another, but individual reference to them is not made except where circumstances demand it.

Linear measurements and fruit volumes. Fruit volumes were measured by displacement of water in measuring cylinders. The relation between volume and certain linear measurements were used as a means of estimating volumes without detaching fruits from the bunch. Measurements of volume, V (c.c.), of length, L (cm.), and mid-circumference, C (cm.), were made on a number of ovaries and fruits of various age and botanical origin. Relations between V and LC^2 were: correlation coefficient, $r = 0{\cdot}9953$, and $V = (0{\cdot}3537 + 0{\cdot}0616\, LC^2)$ c.c. Evidently LC^2 gives a reasonably accurate measure of volume, and this was the method followed in tracing the development of individual fruits. Scratches were made at intervals along the fruit with a needle-point and lengths measured from them to the nearest millimetre by means of dividers; circumferences were measured with pieces of fine thread. The scratches, once made, were permanent as a result of the fortunate accident that wounded banana tissues darken rapidly in contact with air.

In the experiments which form the basis for most of the results a group of six ovaries in the middle of one hand (nodal cluster of flowers) was used; three were pollinated and three were not, treatments being applied at random. Pollen was applied in plasticine capsules as described by Simmonds (1952*a*), the pollen chosen being the one most nearly related to the clone under experiment. Thus *acuminata* pollen was applied to clones of *M. acuminata*, *balbisiana* to *M. balbisiana*, and a choice (often more or less arbitrary) was made in the case of hybrids.

The application of hormones. Attempts to inject ovaries with a hypodermic syringe were unsuccessful since rotting almost always occurred. The method finally adopted was the painting on of a lanolin emulsion made up after the manner of Winklepeck and McClintock (1941).

RESULTS

The developmental anatomy of parthenocarpy. This was examined in transverse sections of fruits of the edible diploid banana, 'Pisang Lilan', which is a parthenocarpic strain of the Selangor form of *Musa acuminata* (Dodds and Simmonds, 1948*b*). A full anatomical study was not undertaken, attention being concentrated on the general course of the development of edible parenchyma and not on vascular or skin anatomy.

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[1] Much work remains to be done on the origins of edible varieties. Unpublished information suggests that the origins attributed to certain varieties in this paper (and in Dodds and Simmonds, 1948*a*) present an unduly simplified picture of the true situation but one which must suffice for the present purpose.

The receptive ovary (i.e. that borne by an open female flower ready for pollination) consists of a firm green vascular skin enclosing three loculi which meet at a central ovuliferous axis. Each loculus is roughly triangular in section and is bounded at its outer face by a zone of soft, white, loosely parenchymatous cells which extends down the septa towards the axis. At the outside, of course, this zone (which will hereafter be called the lining) effectively forms the innermost zone of the pericarp; it is bounded at its internal face by a skin of small nearly isodiametric cells and at the outside grades into the surrounding tissues. Each loculus bears two rows of ovules embedded (or nearly so) in a strip of mucilage which is pervaded at its axial face by numerous hair-like cells emergent from the axis especially in the neighbourhood of the ovules. Vascular bundles are longitudinally disposed in the skin and axis and run radially from the latter through the septa and tangentially across the lining. The ovules begin to degenerate in a week after receptivity and after 2 weeks have completely shrivelled up, leaving the funicles with conspicuously swollen tips. These swollen tips increase in size and persist up to maturity, though often concealed by overgrowth from the surrounding tissues. The numbers of hair-like cells in the mucilage seems to increase for the first week or two, but later all trace of them disappears as the mucilage goes.

In about 3 weeks, growth of the lining becomes apparent, mostly in the outer (i.e. inner pericarp) region but also a little in the septa and axis. The innermost layers of the lining are those that grow, the outer layers remaining loosely parenchymatous up to maturity when they form an easily broken zone which permits peeling of the ripe fruit. The tissue formed from the lining is more densely parenchymatous than the tissue from which it derives and, by continued growth and accumulation of starch grains, finally fills the fruit with edible pulp. No mitoses were observed, so presumably increase in bulk is at least mainly due to cell enlargement. Vascular bundles and latex cells are absent from the edible pulp. At about 8 weeks the locular cavity has almost disappeared and the mucilage and ovule relicts are compressed between the faces of the growing pulp and the slightly swollen axis. From this time until maturity (at about 16 weeks) there is little or no anatomical change in the fruit though there is a great increase in bulk. Signs of internal strain may sometimes be seen; in particular there are indications of tangential strain in the outer parts of the pulp and sometimes cells appear to be broken by it. In some varieties (though not conspicuously in 'Pisang Lilan') the mature green fruit is in a mechanically unstable state in which the pulp seems to have outgrown the skin; very slight damage to the skin causes an almost explosive longitudinal splitting down to the axis. It is interesting that this behaviour was figured by Ehret in 1750 in the type drawing of *Musa sapientum* L. (Cheesman in Dodds and Simmonds, 1948*a*).

Gross development and growth curves of the banana fruit

1. *Seeded diploids.* Growth curves were constructed from weekly measurements of length and circumference. Figs. 1 and 2 illustrate behaviour of two seeded diploid species, *M. acuminata* (clone Calcutta 4) and *M. balbisiana* (clone Ceylon). The growth in volume of pollinated fruit is, in each case, sigmoid and there is a marked initial period of induction lasting 1 to 2 weeks. Length and circumference follow roughly similar tracks, though the two species differ in that *M. balbisiana*, with its plump mature fruit, shows a much greater growth in circumference than the more slender *M. acuminata*. In both cases unpollinated ovaries swell slightly and persist in a green, unwithered condition for about 4 months; in each one of six cases examined growth in the first week was greater than in any subsequent week. The effect of pollination on fruit volume is clearly perceptible 1 week after receptivity. The much greater mature volume of *M. balbisiana* in comparison with *M. acuminata* is correlated with differences in the seed content of the two fruits: thus the fruit of Ceylon, which is about 4 times larger than that of Calcutta 4, contains nearly 3 times as many seeds and 5 times as much seed material by volume.

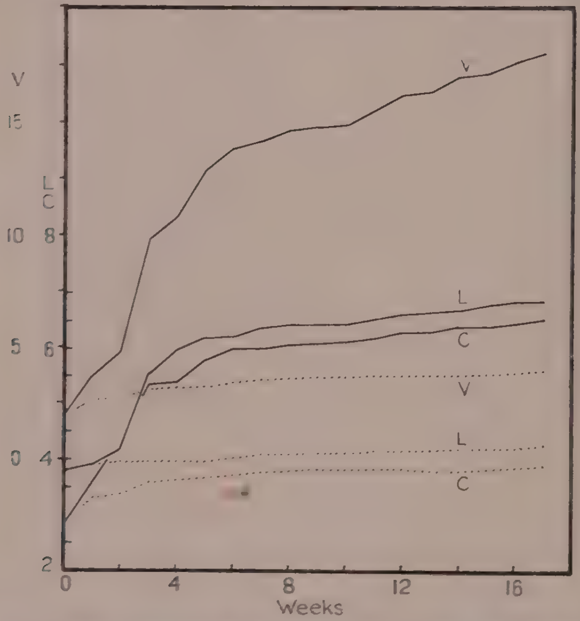

FIG. 1. The development of a seeded diploid banana fruit, *Musa acuminata*, clone Calcutta 4. Means of three pollinated and three unpollinated fruits. *V*, volume (c.c.); *L*, length (cm.); *C*, circumference (cm.); solid lines, pollinated; dotted lines, unpollinated.

2. *Unpollinated parthenocarpic bananas.* Growth of unpollinated parthenocarpic bananas, by contrast with the seeded diploids, is much less constant

and characteristic. 'Gros Michel' (Fig. 3), an *acuminata* triploid, gives a concave curve of volume growth and, in this instance, somewhat irregular linear increases, with length consistently greater than circumference. Bluggoe (Fig. 4), a *balbisiana* triploid, has a convex curve and near equality of length and circumstances. In no case among several scores of similar graphs examined does the volume curve of a parthenocarpic banana resemble the sigmoid curve characteristic of the seeded types. The variety of relation between length and circumference is considerable, indicating that comparable mature fruit shapes may be attained by different developmental tracks.

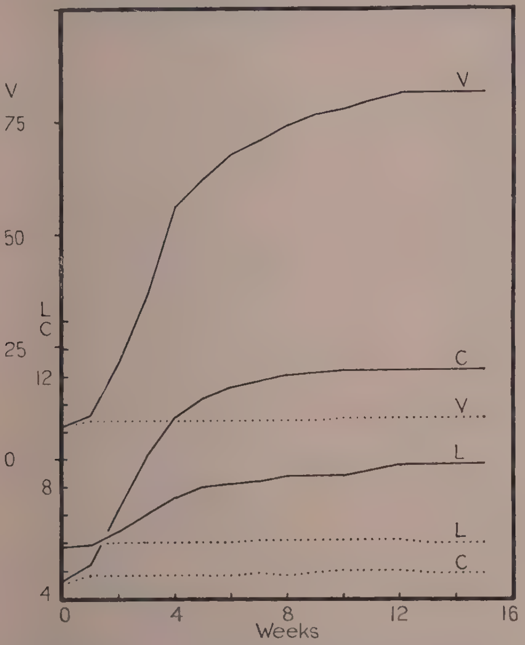

FIG. 2. The development of a seeded diploid banana fruit, *Musa balbisiana* clone Ceylon. Conventions as in Fig. 1.

'Gros Michel' (Fig. 3) has a concave volume curve which, however, starts irregularly; growth in the first week is greater than in the following week. In many other similar graphs the line was either concave or nearly straight and in each case the same check in development was detected at the first or second week, exceptionally (the varieties 'Bande' and 'Marathuva') at the end of the third week. Sometimes, the growth rate in the second week was as little as one tenth of that in the first.

'Bluggoe' (Fig. 4) represents a rather different type of development, for the curve is markedly convex. A number of other similar curves agreed well with it; all showed an early growth rate which was either essentially constant or which showed at most a very slight check after 1 week; thereafter the rate fell slowly and the curve was convex. These behaviours are correlated, as shown below, with those of the ovules.

The distinction between the two main types of development is fairly easy, for one has a concave or roughly straight growth curve with an early growth check and the other has a convex growth curve with little or no growth check. An attempt to subdivide the first type into 'concave' and 'intermediate' gave a consistent arrangement, though the process is admittedly liable to subjective errors. It is concluded that the type of development is characteristic of a clone and, furthermore, that there is a close connexion with specific origins. Examination of forty-nine clones (for most of which two experiments and therefore two growth curves were available) showed that edible clones derived from *M. acuminata* are predominantly concave, those from *M. balbisiana* convex, and those from the hybrids intermediate.

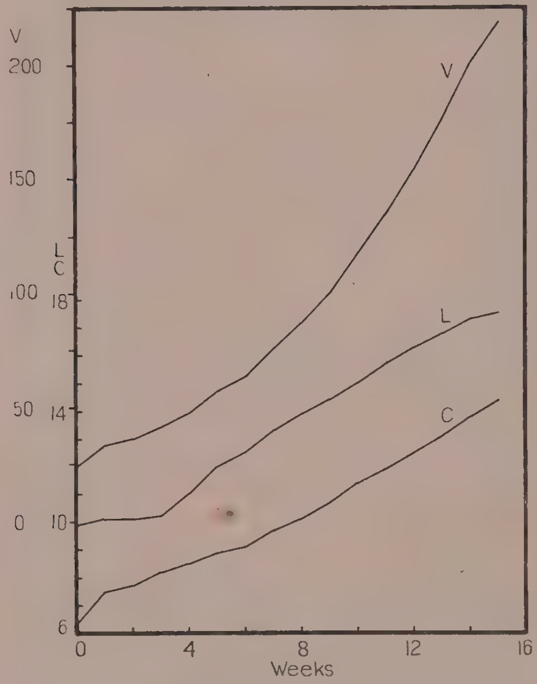

Fig. 3. The development of an edible triploid banana fruit, 'Gros Michel' (*Musa acuminata*). Means of three unpollinated fruits; conventions otherwise as in Fig. 1.

3. *Pollinated parthenocarpic bananas.* The behaviour of pollinated parthenocarpic bananas depends largely on whether seeds are set or not. If seeds are not set, pollination has, at most, a very slight positive effect on fruit size. If seeds are set, they have an easily detected effect on volume proportional to their

umber. These relations are subjected to detailed analysis below. For the resent it is sufficient to refer to Fig. 5, which shows the effect of an average f 6·5 seeds per fruit on the development of 'Awak Legor', a rather fertile *albisiana* triploid. The effect of seediness can be detected in the second week f development and thereafter the pairs of lines diverge steadily. At maturity, olumes of pollinated and unpollinated fruits differ by about 15 c.c., indicating a mean effect of about 2·5 c.c. per seed. The curve, in this and other similar cases, shows no tendency to resemble the sigmoid form typical of seeded bananas; seediness changes the slope of the growth curve but not its form.

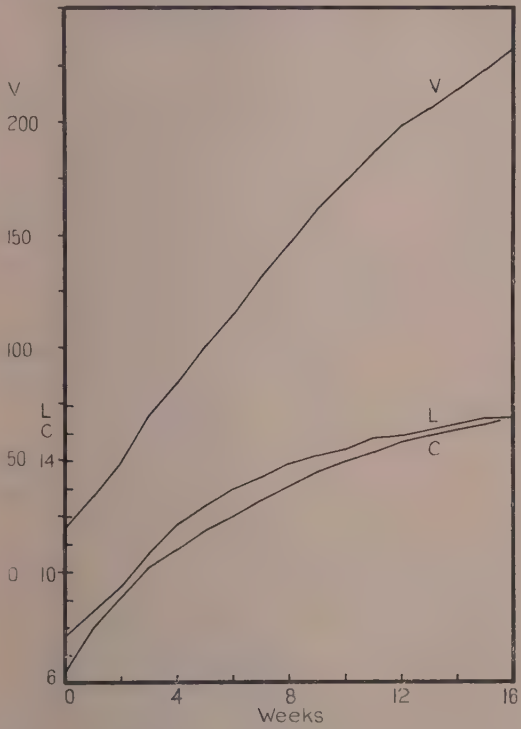

FIG. 4. The development of an edible triploid banana fruit, Bluggoe (*Musa balbisiana*). Means of three unpollinated fruits; conventions otherwise as in Fig. 1.

The effects of pollination

1. *Pollination of seeded bananas.* We have seen above that the development of the fruit of a seeded banana is conditional upon pollination and that the ultimate fruit size attained depends, at least in part, upon the number of seeds. The quantitative relations of fruit size and seed content were examined

by the analysis of data on the effect of different pollens upon seed yield of seeded diploid bananas (Simmonds, 1952*a*). Mature fruit volumes were available for these experiments and the analyses take the form of regression calculations.

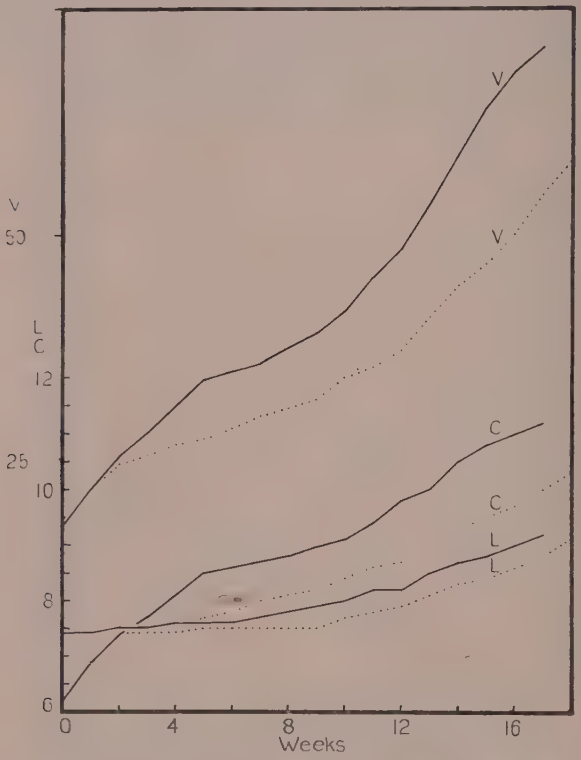

FIG. 5. The development of a rather fertile edible triploid banana fruit, 'Awak Legor' (*Musa balbisiana*). Means of three pollinated and three unpollinated fruits; conventions as in Fig. 1.

In an experiment on *M. acuminata*, clone Calcutta 4, fifty-nine observations of volume and good and bad seeds gave the following multiple regression: $V = 5{\cdot}79 + 0{\cdot}23\,G + 0{\cdot}15\,B$; or $V = 5{\cdot}79 + 0{\cdot}23\,(G + 0{\cdot}66\,B)$, where V = volume in c.c., G = number of good seeds, B = number of bad seeds. The least number of the value $(G + 0{\cdot}66\,B)$ recorded in this experiment was 32 in a fruit of volume 12 c.c., so that the range did not approach the lower limit. But, over the range that was covered, there was no appearance of departure from linearity.

In *M. balbisiana*, clone Ceylon, data were available from 56 fruits in one experiment. The range covered was greater than in Calcutta 4 since some fruits contained no seeds and the largest contained about 300. There were very few bad seeds set in this experiment (only four fruits contained any at ll). The variate $S = G+0{\cdot}66\ B$, as calculated above, was used and it was ound that $V = 9{\cdot}08+0{\cdot}24\,S$. As before, the line was apparently straight over he range studied. However, in the case of the few fruits that lacked seeds ltogether, it was evident that their volumes (5–8 c.c.) were slightly less than he corresponding volume on the regression line (9·08 c.c.); perhaps, then, here is a very slight downward curvature of the line near the origin.

It appears therefore that seed content and fruit volume are linearly related over all but the lowest part of the range of seed numbers; that bad seeds have a considerable effect on fruit volume; that volume increments per seed are virtually identical in the two species; and that the seeds have a pronouncedly stimulative effect on fruit development since the regression coefficients are 3 to 5 times greater than the volume of the seed itself.

2. *Pollination of parthenocarpic bananas.* Many parthenocarpic bananas are slightly seed fertile when pollinated and the effect of seediness on the growth curve has been illustrated above (Fig. 5). Figures are available of volumes and seed contents of mature pollinated and unpollinated fruits for a number of different parthenocarpic bananas. The difference in volume between pollinated fruits and unpollinated controls in the same hand has been related to seed content[1] of the pollinated fruits. The regression line is $D = 4{\cdot}11+1{\cdot}35\ S$, where D is the difference in volume (c.c.) between pollinated and unpollinated fruits. More detailed analysis gave no evidence of curvilinearity of regression. The line is sensibly straight but it does not pass through the origin ($t\ (54) = 3{\cdot}19$); this means that pollinated but seedless fruits are larger by about 4 c.c. than unpollinated fruits.

The magnitude of this regression coefficient (1·35 c.c. per seed) may be compared with those determined for seeded bananas (0·23 and 0·24 c.c. per seed). It is nearly 6 times larger; evidently a seed has an enormously greater stimulative effect in a parthenocarpic than in a seeded maternal environment.

The finding that seedless pollinated fruits are significantly larger than the seedless unpollinated controls was further examined in four experiments designed for the purpose. The edible triploid clones 'Mysore' and 'Rajapuri' were chosen; pairs of adjacent ovaries were used as experimental units and two bunches of each clone were treated. One member of each pair of ovaries was pollinated, the other was not, treatments being applied at random. At maturity volumes were measured and seeds counted. All four experiments showed differences in favour of pollinated but seedless fruits, though of these none were large and only one was significant. (Seed-bearing fruits were few and were excluded from analysis.) Classification by hands, however, reveals that the results are heterogenous; pollination has a greater effect in the top hands than in the lower ones. It is known (unpublished) that pollinated 'Gros Michel', an edible triploid, when it bears seeds at all, bears most of them in the top hands of the bunch. Thus it seems that pollination has a stimulative effect on fruit growth independently of recognizable seed set and that this effect is greatest in the upper hands where seeds, if any, are normally borne. This is most probably due to stimulation by fertilized ovules which do not go far enough in development to give rise to recognizable seed at maturity; though it could be due to a stimulatory effect of pollination as such, e.g. by the agency of hormones introduced in the pollen. The former seems much more likely, since we know that 'Mysore' and 'Rajapuri' do sometimes bear seeds after pollination and the correlation between place of stimulation and place of seed set is striking. If this is correct, then similar experiments on highly female-sterile clones should show no effect at all of pollination. This was so in four experiments, two each on 'Pisang Lilan' and 'Robusta' (a triploid related to the Cavendish banana); all mean differences were small, positive or negative, and non-significant, and there were no perceptible differences between hands.

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[1] $S = (G+0{\cdot}5\ B)$ was used; the substitution of $(G+0{\cdot}66\ B)$ as determined above would make little difference to the results which have not, therefore, been recalculated.

Factors affecting ovary and ovule sizes

1. *The effects of ploidy on growth rate.* Volumes of mature fruits were measured (by displacement) at the end of each experiment in which weekly linear measurements were made. Volumes of receptive ovaries were estimated from the linear measurements, using the relation between volume and LC^2 established above. The time of development being known, the mean weekly increment of volume over initial volume could be calculated; results are given in Table I. Figures for receptive ovary volume, since they are estimated rather than measured, are not very accurate, but they do indicate quite clearly that triploid and tetraploid ovaries are about twice as large as those of diploids. Likewise, triploid and tetraploid growth rates are similar one to the other but rather more than twice as great as diploid rates and the difference is highly significant. Growth rates per unit receptive volume are roughly constant. Thus ploidy has a marked effect on rate of growth and hence on ultimate fruit size. Triploid and tetraploid fruits, in fact, at all stages of development are about twice as big as diploid fruits at the corresponding stage.

TABLE I

The relation between ploidy and rate of fruit development in unpollinated parthenocarpic bananas. Growth rates as mean weekly volume increment over receptive ovary volume. In parentheses—numbers of variates

	Ploidy		
	Diploid	Triploid	Tetraploid
Receptive ovary volume (c.c.)	9·0	19·3	19·1
Growth rates[1] (c.c. per week)	2·94(20)	7·22(37)	6·91(20)[1]
Standard errors ±	0·606	0·445	0·606
Growth rate/receptive volume	0·327	0·374	0·362

[1] Analysis of variance: Variance ratio (Ploidy, $n_1 = 2$, $n_2 = 74$) = 17·68***.

2. *Ovule sizes in relation to parthenocarpy.* Preliminary experiments indicated that, in seeded bananas, the ovules of unpollinated ovaries showed a slight increase in size up to 9–14 days, when shrivelling became apparent;

pollinated ovaries, by contrast, contained ovules that grew very much more rapidly and at an ever-increasing rate up to 20 days which was as far as observations were carried. Edible bananas showed a slight increase in size of both pollinated and unpollinated ovules up to about a week when growth ceased and shrivelling started. In pollinated ovaries a few ovules which swelled more rapidly and remained unshrivelled for 2 or 3 weeks had evidently been fertilized. Probably it is ovules of this kind which, failing to complete development into seeds, are responsible for the apparently stimulative effect of pollination without seed set.

Systematic observations (at receptivity and after one week) of a range of diploid and polyploid, seeded and edible bananas are summarized in Table II. Samples of ten ovules each were measured under the microscope and results transformed to mean ovule diameters in millimetres.

TABLE II

Ovule sizes in bananas in the first week of fruit development

			Ovule diameters (mm.)			Increase % of initial size in first week	
Plants	Treatment	Nos.	Initial size	Size after 7 days	Increase	Ovules	Ovaries[1]
Seeded diploids	Pollinated	13	0·93	1·59	0·67	72	43 (6)
	Unpollinated	13		1·05	0·12	13	22 (6)
Edible diploids	Unpollinated	18	1·06	1·59	0·53	50	49 (10)
Edible triploids	Unpollinated	27	1·10	1·61	0·51	46	54 (20)
Edible tetraploids	Unpollinated	17	1·20	1·78	0·58	48	44 (10)
Variance ratios (n_1, n_2)			12·0***(3, 71)	—	18·9***(4, 83)	—	—

[1] Calculated from samples of linear measurement data. In brackets—number in sample.

Edible diploids have larger ovules at receptivity than seeded diploids. Ploidy among edible types evidently affects ovule size, tetraploids being larger than diploids and triploids. After 7 days the greatest increase in size is found in pollinated seeded types, the smallest in the same types unpollinated. All three categories of edible bananas show a roughly intermediate increase in size, significantly less than in the pollinated seeded class but more than in the unpollinated seeded types.

The effects of ploidy on receptive ovule size is to be expected from our knowledge of its effect on cell size and is unimportant for the present purpose. What is significant here is that edible bananas have larger ovules initially than seeded ones and that there is a considerable autonomous increase in ovule size in them that is independent of fertilization. This is evidently one of the stimulative effects of parthenocarpy and one which starts to operate some time before the ovary is exposed to pollination in the receptive state. It is the earliest symptom of parthenocarpy yet detected in bananas. The seeded bananas also show an autonomous increase in unpollinated ovule size which, however, is much smaller than in edible types.

These ovule behaviours are correlated with early fruit growth. Thus unpollinated seeded types and also edible bananas having a detectable early check in ovary growth show parallel autonomous increases in ovule and fruit size in the first week or two of development (Table II); when the ovules shrivel the growth rate of the ovary drops. This suggests either that the ovules are the seat of some process stimulatory to ovary growth and that when they shrivel the process ceases; or that both ovule and ovary growth at this early stage are controlled by a common process and that shrivelling and growth check are but symptoms of the same cause. Pollinated seeded bananas, by contrast, suffer neither cessation of ovule growth nor a check to ovary growth so that fertilization evidently ensures the continuance of the stimulatory process. It should be noted, however (Table II), that the correspondence between ovule and ovary growth in the first week is not very exact. Edible derivatives of *Musa balbisiana* are anomalous in the sense that their ovules shrivel at about the same time as those of other edible bananas, but their ovaries show little or no corresponding growth check. Furthermore, their early rates of ovule growth are perhaps rather lower than those of other edible bananas though rates of ovary growth are much the same. This suggests that the initial stimulus deduced above is small and is masked by the early onset of a subsequent stimulus of different origin.

The induction and inhibition of parthenocarpy

Three experiments are reported here, each one a randomized block, five successive hands of the bunch being used for replication. Treatments were begun at receptivity (i.e. as soon as the flowers were exposed) and continued to maturity or as soon as any fruit showed signs of ripening. Unwanted ovaries were removed in order to allow access to the treated ones. Unpollinated stigmas were protected with the same type of paper-plasticine capsule that was used to apply pollen (Simmonds, 1952*a*). The lanolin emulsions were applied to the whole surface of the fruit.

Induction and stimulation of parthenocarpy with α-naphthylacetic acid. Two experiments were performed, one on a seeded diploid *Musa balbisiana* (clone Ceylon), the other on a weakly parthenocarpic but rather seed-fertile diploid *M. acuminata* (J. 349) derived from the cross 'Gros Michel' (edible triploid) × *M. acuminata* (seeded diploid). There were six treatments: α-naphthylacetic acid (1000 p.p.m.) applied twice a week, once a week, once a month, and once only (at receptivity) to unpollinated ovaries; control 1 (untreated and unpollinated); and control 2 (untreated but pollinated). Two treated fingers in the experiment on 'Ceylon' showed signs of ripening after 9 weeks so this experiment was reaped; the seeds in the pollinated controls were hardening and would have been mature in another 3 weeks and the fingers which bore them had probably attained nearly full size. The experiment on J. 349 was reaped after 10 weeks' development.

Results are shown in Tables III and IV. α-Naphthylacetic acid induced parthenocarpy in the seeded banana and markedly increased fruit size in the parthenocarpic type. In both cases the more frequent applications were the most effective. In the seeded banana induced parthenocarpic fruits were only about half as big as pollinated fruits. By contrast, in the weakly parthenocarpic J. 349, treatment produced fruits about as big as the pollinated and seeded ones though it did not stimulate pulp growth as much as it stimulated the development of the pericarp; hence parthenocarpy was incomplete and the pulp was gappy—indeed the largest treated fruits, which were about 50 per cent. larger in total volume than the unpollinated controls, were judged to contain only about the same amount of pulp as they did.

TABLE III

Induction of parthenocarpy by the application of α-naphthylacetic acid to the ovaries of a seeded diploid banana (M. balbisiana, *clone Ceylon*)

Treatment				
No.	Hormone applications	Pollination	Fruit volume[1] (c.c.)	Remarks
1	2 per week	no	25·2	Parthenocarpy complete, rarely incomplete and nodular. Pulp peripheral in origin, white and tasteless.
2	1 per week	no	21·0	Less fully parthenocarpic than 1.
3	1 per month	no	12·0	Ovaries swollen but not at all parthenocarpic.
4	1 only	no	9·0	Ovaries probably swollen, not at all parthenocarpic.
5	none	no	7·0	Typical unpollinated ovaries.
6	none	yes	51·0	Typical seeded fruits.

[1] Analysis (excluding treatment 6): V.R. treatments (4) = 79·8***, blocks (4) = 1·3; least significant difference between means (5%) = 2·69 c.c.; coefficient of variation = 13·3%. All differences are significant except that between treatments 4 and 5 (which is probably real, nevertheless). Fruits from this experiment are shown in Fig. 6.

TABLE IV

Stimulation of fruit development by α-naphthylacetic acid in a weakly parthenocarpic diploid banana (*J. 349—'Gros Michel'* × M. acuminata)

Treatment				
No.	Hormone applications	Pollination	Fruit volume[1] (c.c.)	Remarks
1	2 per week	no	15·2	Parthenocarpy incomplete, pulp very gappy in nos. 1 and 2, less so in nos. 3 and 4.
2	1 per week	no	14·0	
3	1 per month	no	12·4	
4	1 only	no	10·4	
5	none	no	9·4	Slightly gappy only.
6	none	yes	14·2	Numerous seeds.

[1] Analysis: V.R. treatments (5) = 15·62***, blocks (4) = 2·89*; least significant difference between means (5% level) = 1·7 c.c.; coefficient of variation = 10·3%. Treatments 1–3 exceed the unpollinated control (5) and the more frequent applications produced significantly greater effects than the less frequent.

It is worth adding that preliminary experiments (not reported in detail here) showed that application of the hormone emulsion to one side of the fruit caused parthenocarpic development at or very near the place of application only. Either movement of the chemical was very slow or else it was quickly inactivated. The same sort of localization of effect is seen in the case of developing seeds which stimulate pulp development only in their immediate vicinity.

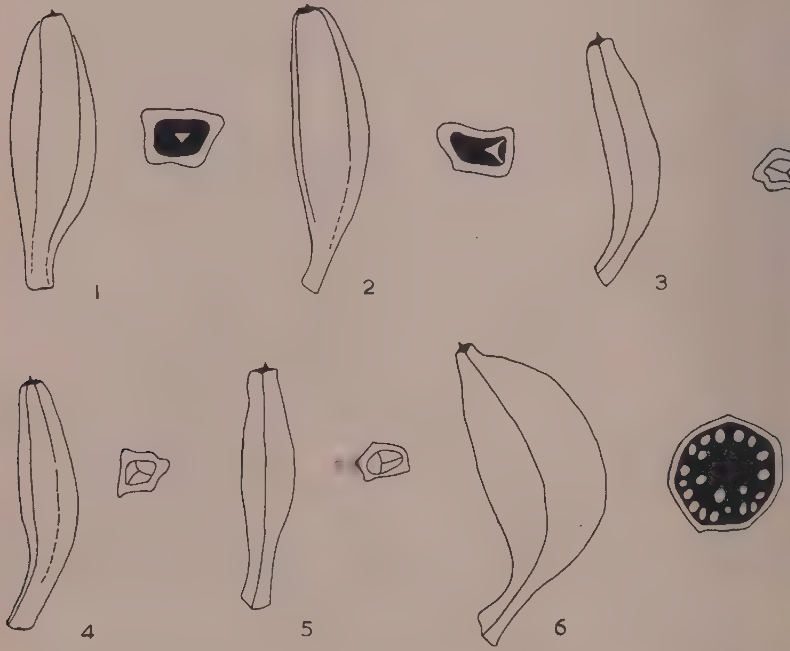

FIG. 6. The induction of parthenocarpy in a seeded diploid banana, *Musa balbisiana* clone Ceylon. Fruits from the first block of the experiment detailed in Table V. Nos. 1–4, α-naphthylacetic acid treatments as in Table V; No. 5, unpollinated control; No. 6, pollinated control. Edible pulp shown in solid black; seeds in No. 6 in white. × 0·43.

Perhaps this localization of effect was responsible for the apparently differential effect of α-naphthylacetic acid on pericarp and pulp, the pericarp being stimulated more readily in these experiments simply because the hormone was applied directly to it.

Inhibition of parthenocarpy with coumarin. An experiment complementary to the two described above was carried out on 'Pisang Lilan', an edible diploid form of *Musa acuminata* that is fully parthenocarpic and highly seed-sterile. Coumarin (1000 p.p.m.) was chosen as a well-known inhibitor of plant growth and it is evident (Table V) that it markedly reduced fruit size. When allowance is made for the volume of skin, one or two applications per week reduced the volume of edible pulp per finger by about 50 per cent. It is a reasonable inference that the coumarin acts by inhibiting a hormone system normally responsible for parthenocarpy.

TABLE V

Inhibition of parthenocarpy by the application of coumarin to the ovaries of an edible diploid banana ('Pisang Lilan')

Treatment				
No.	Coumarin applications	Pollination	Fruit volume[1] (c.c.)	
1	2 per week	no	23·3	Some fruits showed very slight 'gappiness' at the centre.
2	1 per week	no	23·3	
3	1 per month	no	27·8	Fully parthenocarpic.
4	1 only	no	33·5	Fully parthenocarpic.
5	none	no	39·5	Fully parthenocarpic.
6	none	yes	40·3	Fully parthenocarpic and seedless (Pisang Lilan is highly female-sterile).

[1] Analysis: V.R. treatments (5) = 19·8***, blocks (3) = 3·7*; least significant difference between means (5% level) = 5·20 c.c.; coefficient of variation = 11·0%. All coumarin treatments are significantly less than controls and there are significant differences between some coumarin treatments.

DISCUSSION

Fruit development, whether seeded or parthenocarpic, is now generally held to be due to a series of waves of hormone action. This view has been based largely on two approaches to the problem: first, pollen, ovules at the time of meiosis, the developing embryos and/or endosperms have all been shown to be sites of vigorous hormone production (Gustafson, 1939 *a*, *b*, 1942; Witter, 1943); and, second, application of synthetic growth substances to receptive ovaries has, in many plants (though not in all that have been tried), induced parthenocarpy, the implication being that the applied chemical has supplied the hormonal stimulus to growth which, in the absence of pollination and developing seeds, would otherwise have been wanting. The fact that attempts to induce parthenocarpy have sometimes failed is not a very serious objection to the general theory, in view of the recent discovery that synthetic hormones have qualitatively as well as quantitatively specific reactions (Blondeau and Crane, 1948, 1950). Hence mere choice of an experimental chemical might determine success or failure; and, of course, there are also numerous problems of penetration, movement, inactivation, and so forth which might affect the results in any one case.

Contrariwise, natural vegetative parthenocarpy is believed to be due to production of native hormones in the ovary and fruit by tissues which would normally only produce them after pollination; thus Gustafson (1939*a*) has shown that varieties of citrus that are normally parthenocarpic have a higher flower-bud concentration of hormone than varieties normally fertilized and seeded, though it should be observed that this work was not very satisfactory both by reason of the uncertain status of the citrus varieties used in regard to parthenocarpy and the inexactness of location of the auxin concerned. Stimulative parthenocarpy, it may be supposed, differs only in that it needs the pollen hormone to initiate development, perhaps merely to prevent abscission (Gustafson, 1942). Van Overbeek, Conklin, and Blakeslee (1941) made the suggestion that it is not so much the hormone itself in the pollen that is responsible, but rather a critical prosthetic group capable of completing a hormone-liberating enzyme in the ovary. This argument was based on the fact that certain extracts appeared to be far more active in stimulating fruit development than their known contents of indolylacetic acid allowed. However, the recent demonstration by Redemann, Wittwer, and Sell (1951) that the active substance in ethanol extracts of maize kernels is actually ethyl indolylacetate (which is very much more active than indolylacetic acid itself) goes far to resolving the problem.

With these views in mind we may try to outline the main features of fruit development in bananas. The banana fruit is seeded or vegetatively parthenocarpic; there is no evidence for any stimulative effect upon fruit development of pollination as such. Seeded fruits grow at a rate which is virtually exactly proportional to their seed content and each seed secures an increment in fruit size which is roughly four times as great as its own volume; this increment is largely made up of parenchymatous pulp around the seeds and there is therefore stimulative action upon the surrounding maternal tissue. Unpollinated fruits of seeded varieties do not absciss, since there is no abscission layer in the banana pedicel. The slight initial growth they show may reasonably be attributed to hormone production in the ovules at the time of meiosis which takes place a few days before receptivity.

In most parthenocarpic bananas the correlated autonomous growth of ovules and ovaries in the first week is presumably the same phenomenon as that found in the seeded forms; but there is a difference, for at a corresponding morphological stage of development (receptivity) the ovule of the parthenocarpic banana is distinctly larger than that of the seeded type. This cannot be due to differences in internal events, since Dodds (1945) has shown that the development of the female cells in the edible diploid 'Pisang Lilan' is essentially normal. It must be due to the amount (and perhaps to timing of production) of hormone available to the prereceptive ovary. The ovule in the parthenocarpic type must have a greater supply or an earlier one than that of the seeded banana. Subsequent growth of the parthenocarpic banana depends essentially on the same process as occurs in the seeded forms, i.e. the development of a starchy parenchyma from the locular wall. But here the stimulus (though doubtless hormonal in nature, as shown by the experiments with α-naphthylacetic acid and coumarin) is of unknown origin. It cannot come from the ovules since they have shrivelled. The pronouncedly local effect of applied hormone would perhaps suggest that the natural substance is liberated where is has its action, namely, in the inner part of the pericarp. The necessity of repeated application of the chemical stimulant suggests that its effect is not autocatalytic as so often seems to be the case when one or two applications at flowering suffice not only to initiate but also to complete parthenocarpic development (see, e.g., Blondeau and Crane (1950) on the fig; Gustafson, 1939*b*).

The effect of seeds in an edible banana is of interest. They have a stimulative effect on pulp production nearly 6 times greater than in a seeded fruit. Most of the material upon which this conclusion was based was triploid, whereas the seeded bananas studied were all diploid. The comparison therefore is confounded with ploidy, but if we care to assume that triploidy doubles the stimulative effect (as it doubles the growth rate of parthenocarpic fruits), then we are still left with a factor of two as an estimate of the effect of the parthenocarpic as against the seeded maternal environment on stimulation by seed. A stimulative effect of seed on fruit development is, of course, known in many plants (e.g. the date, Swingle, 1928; the plum, Crane and Brown, 1943; the grape, Olmo, 1946) and a searching analysis of this problem seems to the writer to be one of the most useful approaches to the whole question of fruit development.

Genetical implications. Parthenocarpy in diploid strains of *Musa acuminata* is due to a minimum of three dominant genes that are subject to the action of modifiers that affect the amount and rate of development of edible pulp and the degree of seed sterility. The edible diploids are complex genetic structures which owe their nature to a long period of sexual breeding under human selection and their integrity to subsequent clonal propagation (Dodds and Simmonds 1948*a*; Simmonds, 1953). The finding that parthenocarpy is due to hormone action implies that the genetical control is primarily of the hormones concerned.

Human selection must have been directed at two main features of the banana fruit, namely, growth rate (i.e. fruit size) and seed sterility. Both have a bearing on the general discussion of fruit development in bananas, though there are numerous cytological and genetical complexities which need not concern us here. The growth rates of seeded and parthenocarpic diploid fruits are comparable, though fruits of some clones of the latter attain greater sizes than those of seeded types ever can. By contrast, some parthenocarpic genetic segregates bear tiny fruits, a mere 5 or 6 cm. long. Evidently growth rate is under genetical control and has been subjected to the selection normal in ancient cultivated plants. Triploids and tetraploids have far greater growth rates than diploids, and this is evidently a direct effect of ploidy which has, again, been the object of human selection (cf. Dodds and Simmonds, 1948*a*). It is current experience in banana breeding that tetraploids of the origin 'Gros Michel' (edible triploid, female) × *M. acuminata* (edible diploid, male) do not bear quite such large fruits as the triploid female parent, though times of development are similar. Perhaps tetraploids are a little inferior to triploids in this respect as in some others (e.g. Simmonds, 1952*b*).

Parthenocarpy is apparently always associated with some degree of seed sterility which ranges in different clones from moderate (perhaps 70 per cent.) to absolute (100 per cent.), and it is known to be affected by a complex of genetical and cytological causes (e.g. ploidy, hybridity, chromosomal changes, &c.). The question therefore arises: does parthenocarpy, as such, cause seed sterility? The answer depends partly on the definition of parthenocarpy. To use the terms 'seedless' and 'parthenocarpic' as though they were virtually synonymous begs the whole question, for seed sterility then becomes part of the definition of parthenocarpy, a confusion of terms and concepts which makes some of the literature on this subject almost uninterpretable. To define parthenocarpy as the potentiality for development of a seedless fruit (which may or may not be seeded if pollinated) seems to the writer to be a better practice. Defined in this manner, the question posed above must be taken to refer to a fruit which has the minimum genetic apparatus necessary to procure parthenocarpy and none of the cytological and genetical complexities that normally cause seed sterility. There is no direct answer possible to the question in this form; probably, however, the answer should be 'yes' on the *a priori* grounds that correlated genetical response and physiological disturbance to fruit development (in effect, pleiotropy) would surely be expected to cause some seed sterility by disruption of events either before or after fertilization.

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The Absorption of Water by Leaves in Fogged Air

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SUMMARY

No evidence could be obtained for any metabolically assisted absorption of water by tomato leaves from fogged air.

THE writer (1952) recently repeated some of Breazeale's experiments (Breazeale *et al.*, 1950), and while obtaining the same results could get no evidence of actual water secretion by roots. If water is ever forced through the plant in the reverse direction (leaf to root), the writer suggested that this was more likely to be due to leaf activity than to active exudation by the roots.

If leaves actively absorb water capable of being forced out of the plant elsewhere, it should be forced out of the lower parts of a shoot when the leaves are enclosed in a fogging chamber, and in particular into a capillary tube attached to the lower end of such a cut shoot. That is, a 'leaf-pressure' experiment akin to a 'root-pressure' one should yield positive results.

Series I. Tomato plants were grown to approximately 25 cm. high. The shoot was then cut off under water half an inch above soil level and sealed into the fogging chamber in such a way that only a leafless (or, if necessary, defoliated) lower part of the shoot about 10 cm. long passed through the seal in the floor of the fogging chamber into a conical flask plugged at the neck with cotton-wool. The experiment was repeated 22 times and each experiment continued for 3 or 4 days. In every case the flask either remained to all appearances dry or showed only a very slight dew on the walls. No water ever collected in the bottom of the flasks.

Series II. Eighteen replicates of a similar experiment, including some leaves in the flasks, gave similar results.

Series III. The roots were cut off the plants under water and the upper parts of the plants enclosed in the fogging chamber as in Series I. The base of the cut shoot was brought out through the seal in the floor of the fogging chamber and to it was attached a glass capillary tube by means of a rubber connexion. (For convenience the capillary tube and connector, initially full of water, were actually attached before sealing the plant through the floor of the fogging chamber.) After setting up, the connector was slightly compressed and released again to bring the meniscus in the capillary tube to about half-way up the tube. Its position was then noted. This experiment was set up 3 times in sets of 6. Of these 18 experiments, in 4 the meniscus remained stationary, and

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in all the remaining 14 rose very slightly. There was rarely any appreciable movement of the meniscus even after 24 hours. There was in no case any evidence of the meniscus moving downwards, or therefore of water being forced into the capillary tube from the shoot. A further set of 12 experiments also failed ever to detect any downward movement or exudation from the base of the shoot.

Although the leaves are undoubtedly capable of absorbing water for their own turgor from fogged air, the above experiments indicate that at least in the tomato plant in the conditions of these (and probably Breazeale's) experiments they do not by active absorption (or any other process) force water into the stem. The exudations by roots in Breazeale's experiments are shown by the writer (1952) not to be due to active secretion by the roots, and on the present findings are shown also not to be due to any increased hydrostatic pressure within the plant set up in any way during absorption by the leaves.

It seems probable that the results of Breazeale and the present writer are fully explained as flow of water along the existing diffusion potential gradient between soil and atmosphere, unassisted by any unidirectional secretion of water by either leaf or root tissues.

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A Study of the Absorption and Utilization of Phosphate by Young Barley Plants

I. THE EFFECT OF EXTERNAL CONCENTRATION ON THE DISTRIBUTION OF ABSORBED PHOSPHATE BETWEEN ROOTS AND SHOOTS

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SUMMARY

The effect of the external concentration on the distribution of phosphate absorbed by young barley plants was studied in experimental periods varying from 1 hour to 7 days. The experiments were carried out in water culture with radioactive phosphorus as a tracer. The concentration of labelled phosphate supplied to the plants ranged from 0·0003 to over 30 p.p.m. P.

When the external concentration of phosphate is reduced below 10 p.p.m. P, the proportion of the absorbed phosphate found in the shoots is markedly reduced. Plants which have been treated with low concentrations of phosphate lose recently absorbed phosphate to the outer medium when transferred to phosphate-free solutions. Plants treated with higher concentrations lose a smaller proportion of recently absorbed phosphate.

Different balances of other nutrients do not affect the general relationship between the absorption and distribution of phosphate, though quantitative changes occur.

It is concluded that the retention of phosphate in the roots induced by dilute media is a direct consequence of the small quantity of phosphate entering the roots and is not attributable to the effects of other ions which may be simultaneously absorbed.

INTRODUCTION

THERE is ample evidence that the processes of absorption and translocation are closely interrelated in the intact plant; the nutrient content of roots is not only determined by the balance between these processes, but also is itself an important factor determining their rates (Broyer and Hoagland, 1943; Broyer, 1950). Thus an understanding of the factors which determine the rate of removal of nutrients from roots to shoots is essential in the interpretation of the nutritional relationships of intact plants. The results of a number of recent investigations indicate that the metabolism of the plant as a whole may influence the process. Steward, Prevot, and Harrison (1942) suggested that the shoot may provide the stimulus for the removal of salts from the root. In this connexion the findings of Woodford and Gregory (1948) that the decapitation of seedlings reduced nitrate absorption before it affected respiration is of interest. Williams (1948) considered that competition between roots and

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shoots is a factor of importance in determining the distribution of phosphate n wheat plants. Alberda (1948) and Helder (1951) stressed the importance of growth in regulating nutrient absorption. Arisz, Helder, and van Nie (1951) have studied factors controlling exudation in detached roots. A number of considerations suggested that radioactive tracers would be of value in the study of the distribution of nutrients in plants. This technique makes it possible to distinguish the nutrient absorbed during an experimental period from that already present in the plant. Moreover, the sensitivity of the method is such that precise observations can be made over periods so short that weight changes are negligible, and the amount of nutrient absorbed is insignificant by comparison with that originally present in the plant. Thus it is possible to study in plants of *equal* nutrient status the absorption and utilization of widely differing quantities of nutrient. In a study of the absorption of phosphate, particular advantage should accrue from the opportunity tracers provide for using very dilute culture solutions, for example, 0·001 p.p.m. P. The sensitivity of colorimetric methods for phosphate estimation is such that except when very elaborate procedures are used (cf. van den Honert, 1933) it is difficult, especially in short experiments, to make precise observations if solutions contain less than 1 p.p.m. P (3×10^{-5}M. $H_2PO_4^-$). Agricultural considerations indicate the desirability of studying absorption from more dilute solutions since the phosphate content of the soil solution may be less than 0·01 p.p.m. P. and seldom exceeds 3 p.p.m. P (Russell, E. J. and E. W., 1950). Furthermore, such studies might show whether a concentration threshold exists below which phosphate absorption does not occur, as was suggested by Olsen (1950).

In the present investigation two principal questions were selected for prior consideration, namely, the relationship between external concentration and the rate of absorption of phosphate in actively growing plants, and the relationship between the amount of phosphate absorbed and the pattern of phosphate distribution. It has become evident that internal factors which affect the translocation and utilization of absorbed nutrients are of considerable importance in determining the rate of absorption. Thus it is appropriate to consider first the factors responsible for the distribution of absorbed phosphate in plants provided with different quantities of that nutrient. The relationship between external concentration and the rate of absorption will be discussed in a later paper.

EXPERIMENTAL METHODS

Plant culture methods. The experiments were carried out in a greenhouse in an environment controlled to only a small extent. In summer thermostatically controlled fans were employed to reduce the temperature; in winter the temperature was not permitted to fall below 13° C. and the length of the light period was maintained at 12 hours by the use of fluorescent lights. Experiments were carried out throughout the year; growth rates therefore varied considerably between experiments.

A pure line of barley, B.N.8, kindly provided by the Director of the National Institute of Agricultural Botany, Cambridge, was used. The grain was graded for uniformity of size, treated with 0·1 per cent. mercuric chloride for 30 seconds to sterilize the seed coats, and germinated in the dark on trays lined with filter-paper moistened with a phosphate-free nutrient solution consisting of the following quantities of salts expressed as m.eq./l.: Mg SO_4, 3; Ca $(NO_3)_2$, 3; KNO_3, 5; K_2SO_4, 1; and Na NO_3, 2; with ferric citrate, sodium diborate, and Mn SO_4 giving the following total concentrations in p.p.m.: Mg 36, Ca 60, K 234, Na 46, NO_3 620, SO_4 386, Fe 0·31, B 0·058, and Mn 0·25. When the seminal roots of the majority of the grain were approximately 1 cm. long, the most uniform 50 per cent. of the seedlings were transferred to tanks containing the nutrient solution. Aeration was provided through aloxite blocks and the plants were supported between pairs of internally blackened glass tubes separated by suitable distance pieces. This arrangement was preferred to the use of waxed cotton netting to support the seedlings, since injury to roots was greatly reduced when they were transferred to other vessels. When the second leaf was developing, the plants were again graded for size and placed in culture vessels containing 300 or 600 ml. of culture medium. Suitably cut waxed corks were employed to support both the plants and glass aerating devices. Two plants, or in some experiments four, were employed per vessel. The disadvantage of using such small experimental units was realized, but the available facilities made it unavoidable. The resultant variability was reduced as much as possible by the grading of plants and by matching them in blocks according to leaf length. 300 ml. vessels containing two plants were used in all experiments, except when other arrangements are specified. When plants were transferred from one solution to another during the course of an experiment, the cork carrying the plants was removed from the vessel, the roots washed rapidly in distilled water, and the cork then inserted into a vessel containing the new solution, a clean aerator being provided.

It was impracticable to raise the plants in sterile conditions. Fungal or bacterial contamination was, however, reduced by cleaning glassware in acid and subsequently steaming it. Corks were regularly re-waxed. When necessary, insecticidal and fungicidal treatments were employed in the greenhouse. These precautions, combined with the washing of roots when plants were transferred, resulted in no noticeable infection occurring during the short experimental periods employed.

Preparation of labelled phosphate solutions. Labelled phosphate solutions were prepared from A. R. KH_2PO_4 to which was added P^{32} in the form of phosphoric acid prepared at the A.E.R.E., Harwell, by the neutron bombardment of sulphur. The pH of culture solutions was kept in the range 5·5 to 6·5. In each experiment a constant activity of P^{32} was used per litre of culture medium. Thus the proportion of the labelled phosphate which was absorbed could be determined by radioactivity assay with the same accuracy in all treatments, but the accuracy with which a given quantity of phosphate could e measured varied inversely with the concentration of the culture medium. he activities of P^{32} employed were usually less than 5μ C./litre, and it is onsidered that radiation induced no significant abnormalities in the plants. his question has been discussed elsewhere (Russell and Martin, 1952).

From 31·6 to 0·0003 p.p.m. P were used in different experiments. Estimates f the concentrations of phosphate in the most dilute media were subject to rror because of uncertainty as to the exact quantity of P^{31} present in the riginal P^{32} preparations. Unfortunately the relative insensitivity of chemical ssay methods prevented the direct measurement of phosphate in such solu-ons. However, since in this work the major interest centres on the effects of oncentrations differing by a factor of 10 or more, such errors cannot obscure e general trend of the relationship between absorption and translocation.

Phosphate is readily adsorbed onto glass from solutions of pH 5 or above. or present purposes this effect may be regarded as irreversible provided that e pH is not lowered (Martin and Russell, 1950). Significant losses resulted rom this cause in dilute phosphate solutions (i.e. less than 0·3 p.p.m. P). How-ver, in examining the relationship between the amount of phosphate entering e plant and its distribution, this effect can be ignored. It may be noted that dsorption onto glass provided a convenient means for ensuring that no ignificant quantity of phosphate was present as an impurity in phosphate-ree solutions. Losses from stock phosphate solutions were prevented by djusting the pH to below 4.

Harvesting and analysis of samples. Roots and shoots were harvested eparately, the former being washed in running water for 5 seconds. The naterial was dried at 100° C., weighed, wet ashed, and analysed by the pro-edures which have been described elsewhere (Martin and Russell, *loc cit.*). amples of culture media were also analysed. Thus balance-sheets could be rawn up to check the validity of analyses and to assess losses due to adsorp-ion onto the walls of culture vessels.

EXPERIMENTAL RESULTS

The general procedure in all experiments was as follows: young barley lants, at the second leaf stage, were treated with different concentrations of abelled phosphate for periods ranging from 1 hour to 7 days. In some experiments additional treatments during or after treatment with labelled phosphate were superimposed upon this general plan. Prior to the experimental treatment, the plants had been grown in phosphate-free solutions (see p. 110): thus they were of low phosphate status, but adequately supplied with other ions. No visible symptoms of nutrient deficiency were observed.

Except to illustrate specific points, dry weight data will not be considered, since no significant differences in weight occurred between treatments after 24 hours, which was the duration of the majority of the experiments. The results are expressed as μg. of labelled phosphate absorbed per plant. This basis of expression has been preferred to the percentage concentration in the plants, since it is known that the absorbed phosphate was not uniformly distributed in the tissues at the end of the experiments. Very marked accumulation occurred, especially in the low phosphate treatments, both in root and shoot meristems (Russell and Martin, 1952; Russell, Sanders, and Bishop, 1949). Moreover, as will be shown below, the distribution of phosphate between labile and relatively immobile forms varied greatly according to treatment. Mean concentration figures would thus be valueless for the estimation of concentration gradients.

A. *General relationship between external concentration and the distribution of phosphate*

Two experiments are conveniently considered together.

Experiment A. At the commencement of the experiment plants were treated for 24 hours (period I) with eleven concentrations of labelled phosphate arranged in a geometric series, adjacent concentrations differing by a factor of $10^{0\cdot5}$, namely:

31·6 p.p.m. P
10·0
3·16
1·00
0·316 p.p.m. P
0·100
0·0316
0·0100
0·00316 p.p.m. P
0·00100
0·000316

Subsequently plants were transferred to a phosphate-free nutrient solution for 7 days (period II). Four replicates were harvested at the end of period I and two at the end of period II. The primary data are summarized in Table I.

Experiment B. Seedlings similar to those in Expt. A were treated for 24 hours with the following concentrations of labelled phosphate:

31·6 p.p.m. P
1·00
0·0316 p.p.m. P
0·00100

Five replicates were harvested after treatment with labelled phosphate for each of the following periods: 1, 3, and 6 hours. A further four replicates were harvested after 24 hours.

Before considering the relationship between the absorption and distribution of phosphate, it is convenient to consider the extent to which the total phosphate content of the plants was increased during the experiments. Colorimetric analyses of representative samples of seed by the procedure of Ward (1933) indicated that the mean initial phosphate content per seed was 0·145 mg. P and that no significant loss of phosphate occurred during the growth of the plants prior to the experimental treatments. In Table I the amounts of labelled phosphate absorbed in 24 hours by plants in Expt. A are expressed as percentages of the approximate initial content of the plants. When the concentration of phosphate in the external solution equalled or exceeded 0·316 p.p.m. P, a considerable increase in phosphate content occurred, while in the 0·1 p.p.m. P treatment the increase was nearly 10 per cent. Lower concentrations, however, caused the total phosphate content of the plants to be increased by only a very small fraction.

TABLE I

Experiment A. Effect of the external concentration on the absorption, distribution, and subsequent loss of phosphate by young barley plants

Initial external concentration of phosphate p.p.m. P	Period I: 24 hours with labelled P (4 replicates)				Period II: 7 days in phosphate-free solution following period I (2 replicates)			
	Labelled P (μg/plant)		% increase in P content of plant	% depletion of culture solution	Labelled P (μg/plant)		Loss of P in period II	
	Root	Shoot			Root	Shoot	μg/plant	% of amount absorbed in Period I
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
31·6	65·5	97·5	112	3·6	52·4	100·0	10·6	6·5
10·0	35·8	85·6	84	7·9	45·6	79·0	—3·2	—2·6
3·16	30·6	81·3	77	25	49·2	74·5	- 11·8	—10·5
1·00	24·9	61·1	59	63	39·1	44·1	2·8	3·3
0·316	13·5	21·1	24	80	14·6	15·6	4·4	12·7
0·100	10·0	3·26	9·2	86	5·95	4·39	3·0	22
0·0316	3·07	0·371	2·4	83	1·62	0·816	1·0	29
0·0100	0·628	0·0337	0·46	73	0·395	0·107	0·16	24
0·00316	0·127	0·0076	0·093	65	0·0668	0·013	0·055	41
0·00100	0·0229	0·00055	0·016	53	0·0121	0·0012	0·010	43
0·000316	0·00648	0·000025	0·0045	48	0·00323	0·00033	0·0030	45

Table I shows that the distribution of phosphate between roots and shoots changed very markedly between treatments. For example, in the 10 p.p.m. P treatment, the ratio of shoot content to root content was 2·4 at the end of period I, while the corresponding figure for the 0·000316 p.p.m. P treatment was less than 0·004. These wide variations in phosphate distribution, combined with the even wider variations in absorption (from over 150 to 0·01 μg. per plant), make difficult the examination of the data without some rearrangement. The percentage of absorbed phosphate found in the shoots has therefore been employed as an index of phosphate distribution; it is here referred to as the 'transport index'. The relationship between this index and phosphate absorption, expressed on a logarithmic scale has been employed for the graphic representation of the effect of the amount of phosphate entering the plant on phosphate distribution. The general trend of this relationship between adjacent treatments throughout the entire concentration range can be thus demonstrated conveniently.

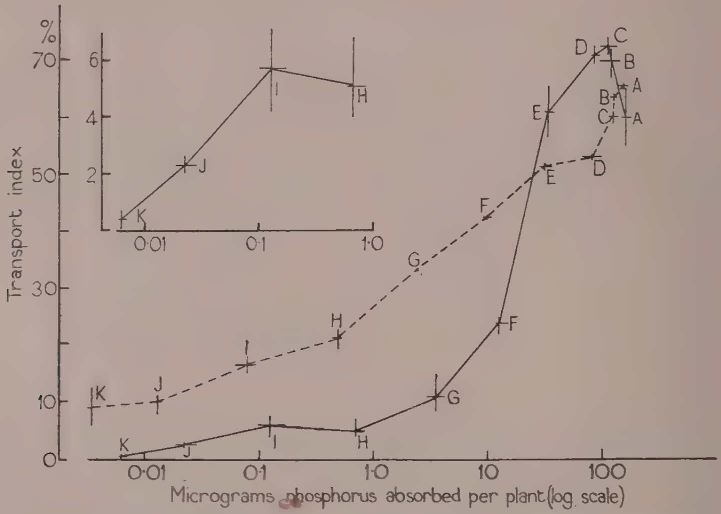

FIG. 1. *Experiment A*: Effect of amount of phosphate absorbed by young barley plants on the transport index. *Solid line*: Plants sampled at end of period I. *Broken line*: Plants harvested at end of period II (see p. 112).
Initial concentration of phosphorus in solution: A, 31·6; B, 10·0; C, 3·16; D, 1·00; E, 0·316; F, 0·100; G, 0·0316; H, 0·0100; I, 0·00316; J, 0·00100; K, 0·000316 p.p.m. P. The range of values at each point is represented by vertical and horizontal lines.

The relevant data for Expts. A and B are shown in Figs. 1 and 2. The treatment means are plotted and in Fig. 1 the range of values for the transport index and for absorption are indicated by horizontal and vertical lines at each point. A detailed statistical analysis of the results has not been undertaken; not only did the nature of the data render a simply analysis of variance or the fitting of regression lines unsatisfactory, but also the magnitude of the principal fects was so great that no mathematical devices were necessary to demon-rate their significance. Statistical procedures have therefore been used only illustrate particular questions.

The same general relationship between the amount of phosphate entering he plants and the transport index was shown in period I of Expt. A (Fig. 1) nd in Expt. B (Fig. 2). When 1 μg. or less was absorbed, the index was ery low; it increased steeply as the amount of phosphate entering the plants

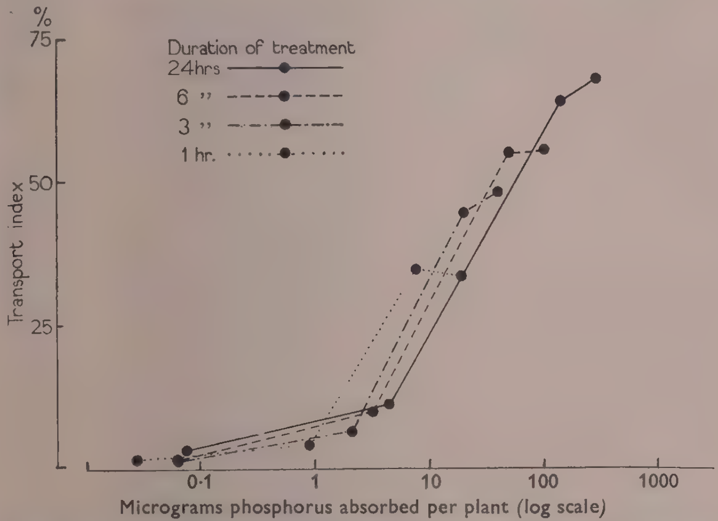

FIG. 2. *Experiment B*: Effect of duration of treatment on the relationship between the amount of phosphate absorbed by young barley plants and their transport index.

ose to 100 μg. Contrasting results were, however, obtained in the two xperiments when the amount of phosphate absorbed exceeded 100 μg. n Expt. A after 24 hours (period I) the transport index attained a maximum f 73 per cent. when the initial external concentration was 3·16 p.p.m. P 111·9 μg. P absorbed per plant) and fell to 59 per cent. at the 31·6 p.p.m. evel (163·0 μg. P absorbed per plant) (Fig. 1). However, in Expt. B, when lants were treated with labelled phosphate for 24 hours, the index rose with ncreasing external concentration over the entire range. The results of Expt. suggest that when absorption exceeds a certain level, 'luxury' accumula-ion in the root may result. That such an effect was more likely to occur in the 1·6 p.p.m. P treatment of Expt. A than in Expt. B is indicated by the fact hat whereas in Expt. A absorption increased by a factor of 1·5 when the xternal concentration was raised from 1·0 to 31·6 p.p.m. P, the corresponding igure in Expt. B was 2·5. This difference may have been due in part to the maller size of plants in Expt. A (mean dry weight per plant: Expt. A—6·6 mg.; Expt. B—80·6 mg.). Further work on this question was not undertaken since the primary object in the present study has been to examine the effects of very low levels of absorbed phosphate on its distribution.

A surprising feature of the results of Expt. B was the close similarity between the curves for the four sampling times (Fig. 2). It appears, therefore, that under these conditions the amount of phosphate absorbed is a factor of greater importance in determining its distribution than the time it has been within the plant. It must be borne in mind, however, that the variability of the experimental material would have prevented the detection of small differences. Expt. A provides more detailed information on some aspects of the changes in phosphate distribution which occurred with time. During period II of this experiment the plants were maintained in phosphate-free media for 7 days. This treatment caused the transport index to rise in plants which had previously been supplied with the lowest concentration of phosphate, while it decreased when higher concentrations (0·316 p.p.m. P or more) had been supplied. When the total contents per plant on these two occasions are compared, it is found that a considerable proportion of the absorbed phosphate was lost from the plants which had previously been treated with low concentrations of phosphate (Table I, cols. 6 and 7). A much smaller proportionate loss occurred from the plants which had been supplied with higher concentrations, although the actual amount they lost was greater. Loss from roots does not, however, account entirely for the changes in the transport index of plants supplied with the most dilute solutions. This is apparent from Fig. 3, in which the contents of roots and shoots are shown for both sampling occasions. When 0·1 p.p.m. P or less had been supplied, the phosphate content of shoots increased at the expense of the roots during period II; this effect was highly significant.[1] Thus it is apparent that when the amount of phosphate supplied in the initial period of the experiment was low, the roots subsequently lost phosphate both to the outer medium and to the shoots. The reverse situation was shown by the 0·316 p.p.m. P to 1·00 p.p.m. treatments, shoot content being reduced at harvest II. The three treatments 3·16–31·6 p.p.m. which, it has already been noted, provided indications of luxury accumulation in the roots, showed no significant change in shoot content.

It is apparent that in Expt. A the concentration range of 0·1 to 0·316 p.p.m. P was critical with regard to the mechanism affecting phosphate distribution. Not only did the transport index change more rapidly in period I between these concentrations than elsewhere, but also the pattern of redistribution of phosphate during period II changed abruptly in this concentration range. It

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[1] In the concentration range 0·000316 to 0·1 p.p.m. P each replicate sampled at the end of period I showed a lower shoot content than either replicate sampled at the end of period II. Four replicates were taken at the end of period I and two at the end of period II, when there was one missing sample. Thus a simple non-parametric test indicates that the probability that the above distribution of values would have occurred by chance is

$$\frac{1}{5}\times\left(\frac{1}{15}\right)^5 \text{ or less than 1 in } 3\times10^6.$$

Non-parametric distributions of this type have been discussed by White (1952).

ould seem that this may be due to the fact that absorption during period I aused little change in total phosphate content of plants supplied with 0·1 .p.m. P or lower concentrations, whereas the phosphate content of plants was ncreased by 25 per cent. in the 0·316 p.p.m. P treatment. It therefore appears robable that the pattern of phosphate redistribution in plants treated with ·316 p.p.m. P or more concentrated solutions was determined both by the irect effect of the amount of phosphate absorbed in period I and by metabolic

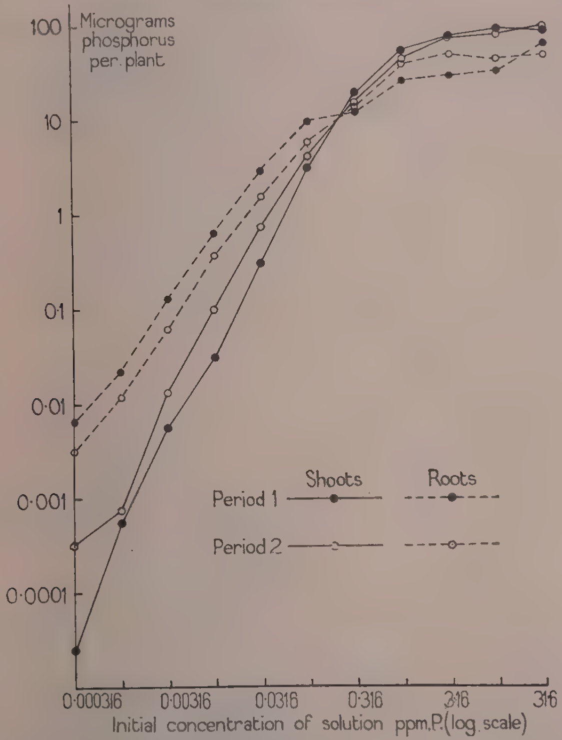

FIG. 3. *Experiment A*: Phosphate content of roots and shoots of young barley plants treated with different concentrations of labelled phosphate for 24 hours (period I) and subsequently supplied with phosphate-free media for 7 days (period II).

differences induced by the increased phosphate status of the plants. When, however, the external concentration was 0·1 p.p.m. P or less, the direct effects of the amount of phosphate absorbed would appear to be the only factors determining differences in the redistribution of phosphate.

Confirmatory evidence with regard to the relationship between the transport index and the amount of phosphate absorbed is provided by a number of experiments designed primarily to examine other aspects of phosphate absorption. Each experiment included treatments in which plants at the second leaf stage were analysed after treatment with labelled phosphate for 24 hours.

The results (Fig. 4) are similar to those obtained in the corresponding treatments of Expts. A and B; very low values for the index were recorded when the amount of phosphate absorbed was small and the index increased steeply with increasing phosphate absorption.

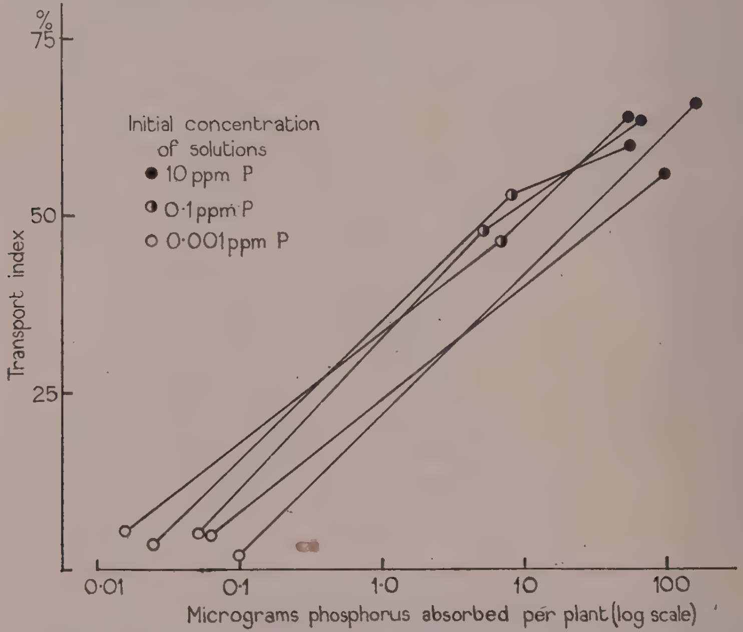

FIG. 4. Effect of amount of phosphate absorbed by young barley plants on the transport index in five experiments.

B. *The relationship between absorption and the outward diffusion of phosphate*

To obtain additional evidence with regard to the loss of phosphate from plants which had previously been supplied with solutions of varying concentration, the amount of phosphate which diffused out of plants was measured in two experiments.

Experiment C. Plants at the second leaf stage were treated for 24 hours with 10, 0·1, and 0·001 p.p.m. P. They were then transferred to phosphate-free nutrient solutions (see p. 110) for a further period of 24 hours. The plants and the solutions were then analysed. Replication was fivefold.

Experiment D. The procedure was similar to that in Expt. C except that the concentrations of phosphate employed were 10 and 0·001 p.p.m. P.

The results for the two experiments are shown in Table II. The amount of phosphate absorbed in the first period of each experiment has been determined by adding the phosphate content of the solutions employed in the second period to the plant content, and the percentage of labelled phosphate which diffused out of the plants in the second period has been calculated. In considering these results it is to be borne in mind that adsorption of phosphate onto the walls of the culture vessels must have occurred in the second period of each experiment; the proportion of phosphate lost in this manner will have been greatest when the content of the solutions was least. Consequently the proportion of absorbed phosphate which diffused out of the plants supplied with 0·001 p.p.m. P will have been underestimated to a greater extent than when high concentrations were applied. None the less, Table II shows that when the plants were supplied with 0·001 p.p.m. P, a significantly larger proportion of recently absorbed phosphate was recovered from the final solution than when higher concentrations of phosphate were applied. This result agrees with the conclusion reached in Expt. A that the proportion of the recently absorbed phosphate which is lost by plants into phosphate-free solutions is greatest when the amount of phosphate previously absorbed is low.

Table II

Experiments C and D. Absorption and loss of phosphate by young barley plants treated with P^{32} *for 24 hours (period I) and transferred to phosphate-free media for a subsequent 24 hours (period II) before analysis.* P *contents and losses are expressed in μg./plant*

Initial conc. medium *Expt. C* (5 replicates)	Content at end of period II	Lost during period II	Absorbed during period I	% of absorbed P lost in period II
10 p.p.m. P	281	<3	283	<1
0·1	11·36	0·163	11·52	1·41±0·41
0·001	0·0685	0·00467	0·0732	6·32±1·13
Expt. D (5 replicates)				
10	126	<1	126	<1
0·001	0·0315	0·00445	0·0360	12·5±1·7

C. *The effect of other ions in the external medium on the distribution of absorbed phosphate*

In Expts. A and B plants were treated withl abelled potassium phosphate in the absence of other salts. Further experiments were undertaken to examine the effects of other ions on the pattern of phosphate distribution. Varying concentrations of ferric iron and nitrate and a balanced culture medium were chosen for this purpose. Since ferric iron is known to have a considerable effect on phosphate distribution (Biddulph, 1948), its interaction with the external concentration of phosphate with respect to the transport index seemed of particular interest. Because nitrate is rapidly absorbed by actively growing roots, it was considered that the use of this ion would demonstrate whether the 'antagonism' of ions present as impurities in the more dilute media was responsible for the relationships shown in the previous experiments. The use of a balanced nutrient solution (p. 110) provided an alternative method of investigating this subject. Moreover, since the solution contained 6×10^{-3} M. potassium, the varying amounts of labelled potassium phosphate used to produce concentrations of from 31·6 to 0·001 p.p.m. P would cause relatively little change in the potassium content of the solution. Thus it could be determined whether variation in the concentration of that ion contributed to the effects demonstrated in the earlier experiments. The likelihood that any such effect would occur was, however, small in view of the ample potassium supply in the solution in which the plants were grown prior to the experiments.

TABLE III

Experiments E and F. Effects of ferric citrate and calcium nitrate respectively on absorption and distribution of phosphate in young barley plants treated with varying concentrations of phosphate. Duration of experiment, 24 hours. When a logarithmic transformation was necessary for statistical analyses, the transformed values are shown in italics

Initial conc. of culture medium *Expt. E.* (5 replicates)		μg. P. per plant			Transport index
P	Fe	Root	Shoot	Total	
10 p.p.m.	2 p.p.m.	62·3	97·5	157·8	60·6
	0·2	42·2	94·3	136·5	68·9
	0·02	36·4	92·3	128·7	71·1
	S.D.	7·6	n.s.	20·9	2·1
0·1 p.p.m.	2	5·57	1·12	6·69	17·43
	0·2	9·71	4·43	14·14	31·15
	0·02	11·28	6·07	17·35	35·28
	S.D.	2·63	0·83	3·05	6·89
0·001 p.p.m.	2	0·0206	0·000036	0·0206	0·19
			0·556		
	0·2	0·0253	0·000053	0·0254	0·21
			0·724		
	0·02	0·0490	0·000295	0·0493	0·33
			1·470		
	S.D.	0·0089	*0·474*	0·0090	n.s.
Expt. F. (6 replicates)					
P	$Ca(NO_3)_2$				
10 p.p.m.	$3\cdot3\times10^{-4}$ M	46·8	85·7	132·5	64·9
($3\cdot3\times10^{-4}$ M)	$3\cdot3\times10^{-8}$ M	47·0	61·5	108·5	56·4
	0	39·9	51·9	91·8	55·8
	S.D.	8·9	17·0	24·0	4·8
0·001 p.p.m.	$3\cdot3\times10^{-4}$ M	0·0711	0·00223	0·0733	2·98
($3\cdot3\times10^{-8}$ M)			*1·348*		*1·474*
	$3\cdot3\times10^{-8}$ M	0·0764	0·00051	0·0769	0·68
			0·708		*0·833*
	0	0·0647	0·00040	0·0651	0·63
			0·602		*0·799*
	S.D.	n.s.	*0·163*	n.s.	*0·152*

Experiment E. The following concentrations of phosphate and ferric iron were combined factorially:

Phosphate:	10	0·1	0·001 p.p.m. P
Ferric iron:	2	0·2	0·02 p.p.m. Fe

Iron was provided as ferric citrate; thus the precipitation of ferric salts in the external solution was prevented. Five replicates were employed and the plants were harvested after treatment for 24 hours. The results are shown in Table III, while in Fig. 5 the relationship between the amount of phosphate absorbed and the transport index is shown graphically. At each level of iron the transport index decreased with decreasing absorption of phosphate in a manner similar to that shown in Expts. A and B. Marked effects of iron were, however, apparent. At all concentrations of phosphate the transport index was depressed by ferric iron. A more complex relationship was apparent with regard to absorption; when 10 p.p.m. P were provided, absorption was somewhat increased at the higher levels of iron, while in the intermediate and low phosphate treatments, absorption was greatest in plants supplied with the lowest concentrations of iron.

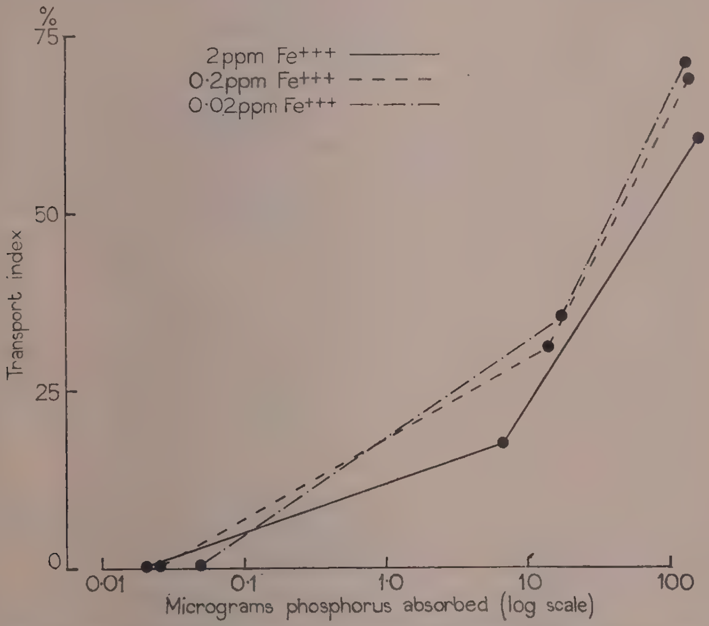

FIG. 5. Effect of ferric citrate on the relationship between the amount of phosphate absorbed by young barley plants and the transport index.

Experiment F. The following concentrations of phosphate and nitrate were combined factorially:

Phosphate: 10, and 0·001 p.p.m. P
(i.e. $3{\cdot}3 \times 10^{-4}$ and $3{\cdot}3 \times 10^{-8}$ M).
Nitrate: $3{\cdot}3 \times 10^{-4}$, $3{\cdot}3 \times 10^{-8}$ M and nil.

Nitrate was provided as the calcium salt. Five replicates were harvested after treatment for 24 hours. The results are shown in Table III.

Calcium nitrate increased absorption to a significant extent in the 10 p.p.m. P treatment; a tendency in this direction which was, however, not significant was apparent in the 0·001 p.p.m. P treatment. The transport index was significantly increased at both levels of phosphate, the effect being considerably greater in the 0·001 p.p.m. P treatment. At all levels of calcium nitrate, however, the general effect of phosphate absorption on translocation index was similar to that shown in earlier experiments.

TABLE IV

Experiment G. The effect of external concentration on the absorption and distribution of phosphate in young barley plants treated for 8 days with labelled phosphate. (*Three replicates*)

Initial concentration of culture medium p.p.m. P	μg. P per plant			Transport index
	Root	Shoot	Total	
5·1	123	450	573	78·5
1·6	57·3	104	161	64·6
0·51	25·5	32·1	57·6	55·8
0·16	8·38	8·38	16·76	50·0
0·051	2·80	2·00	4·80	41·7
0·016	0·908	0·467	1·37	33·9
0·0051	0·303	0·131	0·434	30·1
0·0016	0·084	0·036	0·120	30·1

Experiment G. The following concentrations of labelled phosphate were applied in balanced nutrient solutions: 5·1, 1·6, 0·51, 0·16, 0·051, 0·016, 0·0051, 0·0016 p.p.m. P.

Four replicates were harvested after 8 days. The results are shown in Table IV. The transport index again decreased with decreasing phosphate absorption. The index was, however, markedly greater in the lowest phosphate treatments than in the earlier experiments. Since it was shown in Expt. A that the transport indices of plants supplied with dilute media rose during the 7 days of period II, it appears possible that the higher values for the index in Expt. G were due partially, if not entirely, to the longer duration of the experiment.

DISCUSSION

It is well known that plants which have grown under conditions of phosphate deficiency are characterized by relatively high root to shoot ratios of dry matter (Humphries, 1951) and phosphate content. Williams (1948), who studied these questions in wheat grown under field conditions, sought to interpret them in terms of competition between root and shoot meristems. The present results show the same general effects on phosphate distribution and, furthermore, permit a more detailed analysis of some aspects of the mechanisms responsible. In the simplest analysis the factors which control the distribution within an intact plant of any nutrient absorbed by its root may be divided into three groups: those which regulate the transfer of the nutrient into the conducting tissue of the root; those which determine the rate of upward movement of the nutrient so released; and those which control the rate of downward movement from the shoot to the root. When experimental periods are long, the observed distribution of total phosphate represents the balance between these three groups of factors. Moreover, the interpretation of results is frequently complicated by the effect of phosphate supply on the rate of growth of the plants, which in turn affects the distribution of nutrients. The procedures adopted in the present work reduced the extent of these complications.

Although the highest and lowest concentrations of phosphate applied differed by a factor of 10^5, the phosphate absorbed by the plants treated with the most concentrated solutions for 24 hours was only slightly greater than the seed-borne phosphate already present in them. Over the lower half of the concentration range (i.e. 0·1 p.p.m. P or lower concentrations) changes in the total phosphate content of the plants were trivial (Table I, col. 3). Especially when it is borne in mind that a large part of the phosphate in young barley plants appears to be in the form of inorganic orthophosphate (Heard, 1945), it appears most unlikely that treatments which had little effect on the total phosphate contents of the plants significantly affected metabolism or the movement of phosphate except at the site of entry, i.e. between the root surface and the stele. It may therefore be assumed that the total amount of phosphate translocated between roots and shoots was unaffected. Thus the decrease in the transport index which occurred when the external concentration was reduced below 0·1 p.p.m. P is attributed mainly, if not entirely, to the relatively slower rate at which phosphate was released into the translocation stream. When, however, the amount of phosphate absorbed was sufficient to increase significantly the phosphate content of the plants, it is probable that changes were induced in the translocation of both the labelled phosphate and the phosphate originally present in the plants. It has already been shown that the contrasting patterns of the redistribution of phosphate during period II of Expt. A in plants which had previously been treated with 0·1 and 0·316 p.p.m. P are suggestive of such effects. Changes in the transport index can therefore not be regarded as resulting solely from changes in the rate of release of recently absorbed phosphate into the translocation stream when plant content was significantly altered during the course of the experiments. None the less, the very marked changes in the index which occurred in all the experiments when the phosphate content of the outer medium was raised to 10 p.p.m. P provide strong evidence that the rate of release of recently absorbed phosphate into the translocation stream continued to increase with increasing phosphate absorption. In one experiment it appeared that when the amount of phosphate absorbed was still further increased (i.e. 31·6 p.p.m. P treatment, Expt. A), the rate of release of absorbed phosphate into the translocation stream declined. It is considered that this latter effect may have reflected luxury accumulation;

discussion is here confined, however, to the effects induced by lower concentrations of phosphate.

In the principal experiments here described labelled phosphate was applied as the potassium salt and in the absence of other nutrients. However, in three experiments other ions were applied simultaneously with phosphate, namely, varying concentrations of ferric citrate and calcium nitrate in Expts. E and F, and a balanced culture medium containing 6×10^{-3}M. potassium in Expt. G. Although these treatments affected phosphate distribution, the transport index was in all cases depressed when the amount of phosphate in the culture medium was reduced. Thus it appears that this effect is attributable to the direct effect of the concentration of the phosphate ion and not to the effects of other ions.

The foregoing discussion implies that the rate of equilibration of recently absorbed phosphate with that already present in labile forms in the plant is retarded when the amount of phosphate entering the plants is low. More direct evidence in support of this view is provided by a comparison of the two harvests in Expt. A. The data presented in Fig. 3 suggest that a significant fraction of the phosphate absorbed by plants treated with 0·1 p.p.m. P or lower concentrations was still retained near the site of entry after 7 days, whereas equilibration may well have been complete at the end of the initial period of 24 hours in plants treated with the highest concentrations employed.

Observations of the rate of phosphate loss when plants were transferred to phosphate-free media are of interest in this connexion. It was found that the proportion of the recently absorbed phosphate which diffused from roots into phosphate-free media was markedly greater when the plants had previously been supplied with low concentrations of phosphate. This effect was characteristic of recently absorbed phosphate only; colorimetric analyses indicated no appreciable loss of seed-borne phosphate in plants grown to this stage in phosphate-free media. The outward diffusion of phosphate therefore provides further evidence that when plants were treated with very dilute concentrations of phosphate, a significant part of the absorbed phosphate remained in a different form from the phosphate already present in the plants.

Since phosphate loss was greatest when the plants had been treated with very low concentrations of phosphate, it appeared desirable to consider whether this effect could be attributed to the de-adsorption of phosphate held in a purely physical manner on inert surfaces at or near the cell surface. Were this the case, the low transport indices of plants treated with such solutions might be due largely, at any rate, to physical adsorption. If such a mechanism had been operative, the extent of depletion of the phosphate solutions would have been greatest when the lowest concentrations were applied. Table I, col. 4, shows on the contrary that in Expt. A the greatest depletion occurred in the solutions of intermediate concentration. This effect was consistently shown in all experiments of 24 hours' duration. Moreover, the general form of the relationship between phosphate absorption and the transport index in plants treated with the lower concentrations of phosphate is difficult to reconcile with the hypothesis that phosphate retention in the roots was due to a simple adsorption mechanism. Had this been the case, a significant amount of phosphate must have been retained against upward movement by adsorption in plants supplied with 0·3 p.p.m. P, and it would therefore be expected that a reduction of external concentration by a factor of 10^3 should have completely inhibited upward movement. This did not occur. Since the relationship between the amount of phosphate entering the plant and its distribution between roots and shoots was little affected by the presence or absence of other nutrients, it follows that if this effect were due to adsorption on inert surfaces, the mechanism must have been specific to the phosphate ion. If this were the case, it would not be expected that the outward diffusion of phosphate into phosphate-free solutions would occur to the extent shown by plants which had previously been supplied with low concentrations of phosphate. A further indication that the observed retention of phosphate in roots was not due to a purely physical mechanism is provided by the fact that the immersion of roots in boiling water for 5 seconds reduced the amount of phosphate they subsequently absorbed to approximately the same extent when the external concentration of phosphate was 10 or 0·001 p.p.m. (to 43·5 and 42·0 per cent. of the control values respectively). It is concluded, therefore, that neither the outward diffusion of phosphate observed in these experiments, nor the relationship between the absorption and distribution of phosphate, can be explained in terms of the retention of phosphate by purely physical mechanisms on inert surfaces in plant roots. It appears rather that the outward diffusion of recently absorbed phosphate occurred simultaneously with phosphate entry, the extent of the former process relative to the latter being greatest when the amount of phosphate entering the plant was low. The decrease in the extent to which solutions were depleted when the concentration was lowered below 0·1 p.p.m. P is compatible with such a mechanism.

No suggestions can, however, be made as to whether phosphate loss was due to the outward diffusion of organic compounds in which the phosphate had been retained in the roots or to the release of simple substances, perhaps orthophosphate, following the breakdown of more complex substances. Other workers have shown that phosphate diffuses out of roots in both organic and inorganic forms. Lundegårdh and Stenlid (1944) found that both nucleotides and inorganic phosphate diffused out of growing roots, but not from mature ones. Hevesy (1946) made similar observations using radioactive phosphorus and concluded that one phosphorus atom was lost for every six entering the roots of plants supplied with $0{\cdot}4\times10^{-3}$M. phosphate. It is probable, therefore, that the outward diffusion of phosphate, either in inorganic or organic forms, must be regarded as a normal occurrence in the actively growing regions of roots. Since the present results suggest that recently absorbed phosphate, which has been retained in the roots of plants supplied with low quantities of phosphate, is particularly subject to loss in this manner, it is desirable to postpone discussion of the mechanism responsible for outward diffusion until further information on processes whereby phosphate is retained in plant roots is presented in the second paper of this series.

The experiments with ferric citrate and calcium nitrate are relevant to this discussion primarily because they indicate that the general relationship here established between the amount of phosphate entering the plant and its distribution between roots and shoots is independent of the presence of other ions in the external medium. Both salts, however, influenced phosphate utilization. A high external concentration of ferric citrate reduced the proportion of phosphate which was translocated to the shoots. Similar results were obtained by Biddulph (1948, 1951). His conclusion that the absorption of phosphate is maximal when the external concentration of iron is low appears in the light of the present data to be an over-simplification of the situation. A marked interaction between phosphate and iron with respect to the absorption of the former ion has been demonstrated. When dilute concentrations of phosphate were employed (0·1 or 0·001 p.p.m. P), ferric citrate reduced absorption (Table III). However, when 10 p.p.m. P were provided, the absorption of phosphate was greatest in the plants supplied with the highest level of iron. This is difficult to reconcile with Biddulph's conclusion that the effect of iron on the utilization of phosphate is primarily due to the precipitation of ferric phosphate on the surface of roots and in the conducting tissue. Further evidence that precipitation on root surfaces does not explain the interaction of iron and phosphate is provided by the fact that when the concentration of phosphate is low and that of iron high, the phosphate in roots is largely concentrated in the root-tips (Russell and Martin, 1952) and not distributed evenly over the root surface. Moreover, autoradiographs show phosphate to be distributed throughout the root-tip. No adequate explanation of the interaction of iron and phosphate with respect to phosphate absorption and distribution can at present be advanced, though the low solubility of iron-phosphate complexes may well be an important factor.

In Expt. F the addition of calcium nitrate increased both the absorption and the translocation of phosphate. Whether this was due primarily to the calcium or to the nitrate ion must remain in doubt until further work has been carried out, but since nitrate is more rapidly absorbed than calcium, it appears not unlikely that the nitrate ion was of greater importance. It is usually considered that a high external concentration of nitrate reduces phosphate absorption (Nightingale, 1948). A possible explanation of the present results is, however, suggested by the recent work of Helder (1951), who showed conversely that increasing phosphate supply increased nitrate absorption. Helder interpreted this result as reflecting the effect of phosphate in stimulating growth. The possibility therefore merits consideration that the effects of calcium nitrate, both on the absorption and the translocation of phosphate in the present experiment, reflected increased metabolic activity, especially in the shoots, resultant on increased nitrate content.

ACKNOWLEDGEMENTS

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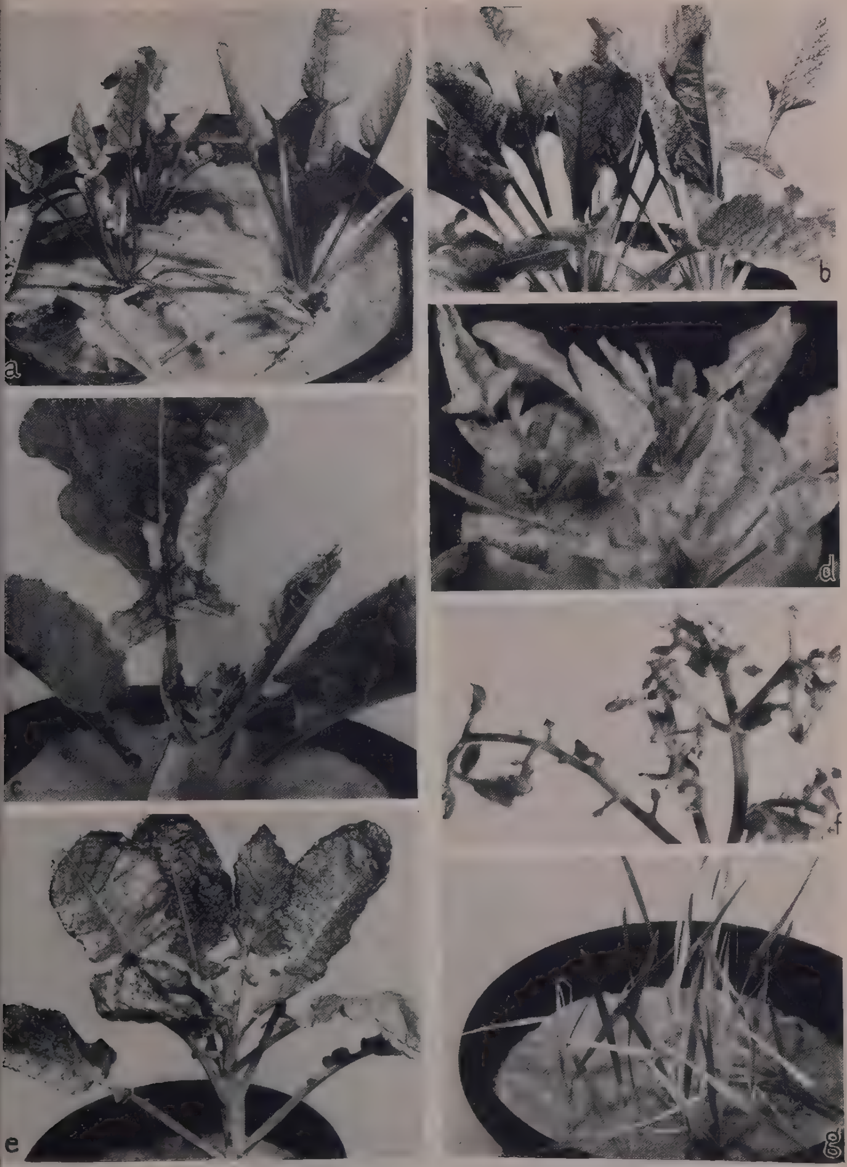
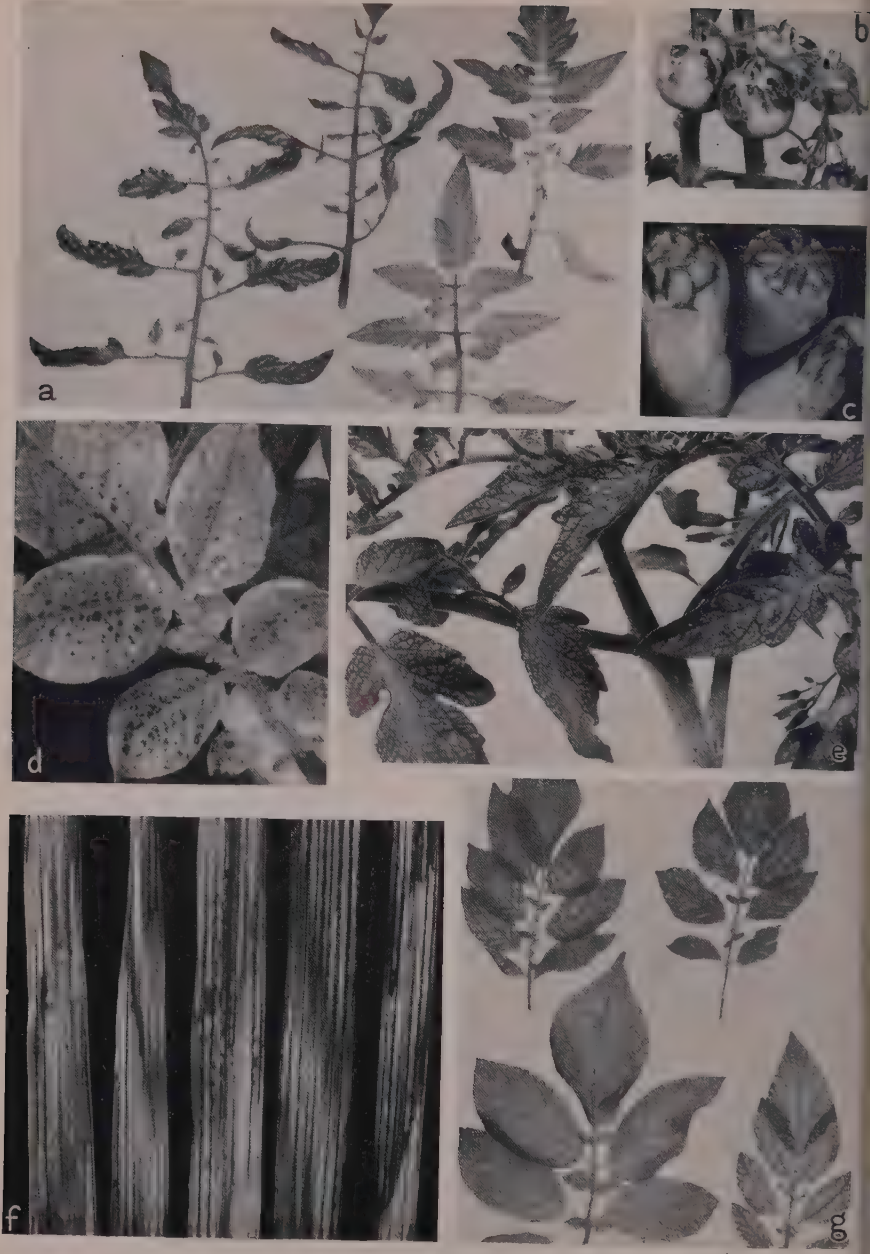


E. J. HEWITT—PLATE I

a. Acute chlorosis in sugar beet from iron deficiency induced by 0·5 m.eq. *L.* CrO_4^{--}. *b.* Moderate chlorosis induced by 0·5 m.eq./*L.* Cr^{+++} under similar conditions. *c.* Nickel toxicity in marrowstem kale; acute chlorosis; scorching, and loss of lamina in young leaves. *d.* Cadmium toxicity in sugar beet; total ivory chlorosis, but absence of necrotic areas. *e.* Cobalt toxicity in marrowstem kale; acute chlorosis; marginal cupping and profuse necrosis. *f.* Cobalt toxicity in tomato; leaflets reduced to peg-like rudiments, severe intervenal chlorosis, abnormal or abortive flower trusses. *g.* Cobalt toxicity in oat; acute chlorosis of young leaves followed by collapse near leaf base.



E. J. HEWITT—PLATE II

a. Left two leaves; Cobalt toxicity in tomato; veins dark green, intervenal areas bright yellow chlorosis and perforated. Lower right centre; Copper induced iron deficiency. Right: Iron deficiency. *b.* Cobalt toxicity in tomato; longitudinal corky splitting of fruit. *c.* Cobalt toxicity in potato; radial or longitudinal corky splitting of tuber at 'rose' end. *d.* Nickel toxicity in potato; chlorosis and rounded black necrotic areas resembling manganese deficiency. *e.* Nickel toxicity in tomato; bright yellow intervenal chlorosis and brown necrotic spotting, veins dark green as in manganese deficiency. *f.* Nickel toxicity in oat; oblique dark green banding alternating with white chlorotic areas. *g.* Cobalt toxicity in potato; two patterns; above, dark green leaves with irregular ivory chlorosis; below, uniform pale yellow intervenal chlorosis.

The Oxidation of Quinic Acid

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SUMMARY

1. It is shown by means of filter-paper chromatograms prepared at intervals during the oxidation of quinic acid by hydrogen peroxide that at least six acids appear in the reaction liquid.

2. One of these acids is shown to be citric acid, and the oxidation of citric acid is shown to account for a further two of the acids resulting from the oxidation of quinic acid.

3. After prolonged oxidation (by H_2O_2) of both quinic and citric acids one acid predominates. This acid is proved by isolation and characterization to be malonic acid.

4. Evidence is produced which suggests that acetonedicarboxylic acid is an intermediate in the oxidation of citric acid (and, therefore, of quinic acid) to malonic acid.

IN the course of the isolation and identification of quinic acid from the young fruits of the Worcester Pearmain apple, filter-paper chromatograms were run at intervals during the oxidation of the quinic acid with hot H_2O_2 (Hulme, 1951). These chromatograms showed that, while the reaction as a whole is a complex one involving at least six acids in addition to the original quinic acid, two acids emerged as the main products of the reaction. One of these was found to be citric acid, which was the acid obtained by Fischer and Dangschat (1934) on oxidation of quinic acid by periodic acid followed by bromine water. The work described in the present paper was carried out primarily to identify the second acid. The results also illustrate the value of paper chromatography in following the course of a complex series of reactions.

The large amount of quinic acid present in young apples—recent work has shown that young Worcester Pearmain apples (average weight 15 g.) contain rather more quinic than malic acid—poses the question of its function in the metabolism of the fruit as indeed in other plant tissues where it is known to exist. For this reason a more complete knowledge of its chemistry is desirable than at present exists.

Small-scale oxidations ($\sim$ 20 mg. quinic acid and 1 ml. 100 vol. H_2O_2) yield, as oxidation proceeds, chromatograms having the patterns shown in Fig. 1. Fig. 2 (upper portion) is a photograph of a chromatogram taken after 25–30 hours' oxidation in the large-scale preparative experiment to be described later; it is included to show the streaking effect produced by the continuous state of flux of the system.

Although it does not follow that the acid-reacting spots on the chromatogram represent a sequence of events commencing at the acid of lowest R_F—during

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the course of a reaction an acid of high R_F may be formed before one of low R_F—oxidation of citric acid by hot H_2O_2 yields chromatograms which reproduce the second stage (citric acid and acids of higher R_F values) of the chromatograms of Figs. 1–2. It will be convenient, therefore to consider the oxidation of quinic acid in two stages, quinic to citric acid and citric to 'X' acid ($R_F \sim$ 0·62).

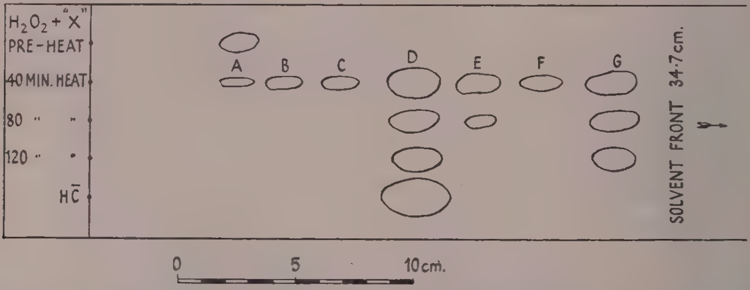

FIG. 1. Chromatogram, run in 'B.F.W.', of the reaction liquid of the oxidation of quinic acid ('X' in the figure) by H_2O_2 (see text). $H\bar{C}$ represents a marker-spot of citric acid.

EXPERIMENTAL

Reagents used. Quinic acid: Purified samples obtained from Worcester Pearmain apples (Hulme, 1951) or commercial samples (Light and Co.; not recrystallized) were used; both gave identical results. *Hydrogen peroxide*: B.D.H., 100 vol. M.A.R. grade was used. Oxidations were carried out in round-bottom flasks fitted with a 'cold finger', in an oil-bath.

Chromatographic methods. Whatman No. 2 paper was used throughout. Down-flowing chromatograms were run in a room kept at a constant temperature of 20° C. All papers were equilibrated for 24 hours with the more aqueous layer (where immiscible solvents were used) before addition of solvent.

Solvents. The two solvent systems used for the detection of acids were butanol-formic acid-water (40:10:50 v/v) due to Lugg and Overell (1948), hereafter designated 'B.F.W.'; ethanol-ammonia-water (80:4:16 v/v) advocated by Long *et al.* (1951) ('E.Am.W.').

The papers were dried in a current of air in an oven at 110° C.

Sprays. With B.F.W., bromo-phenol-blue (5 per cent. in 50 per cent. alcohol, made just alkaline with sodium hydroxide) was used to reveal the position of acids. B.D.H. Universal indicator made to pH 9·5 with soda was found to be considerably more sensitive, revealing the presence of much smaller amounts of acid (Long, Quale, and Stedman, 1951). This spray could, however, only be used with the solvent system E.Am.W. since, even after prolonged drying, papers run in B.F.W. remained sufficiently acid to render the whole of the paper sprayed with the alkaline Universal indicator red almost immediately after spraying.

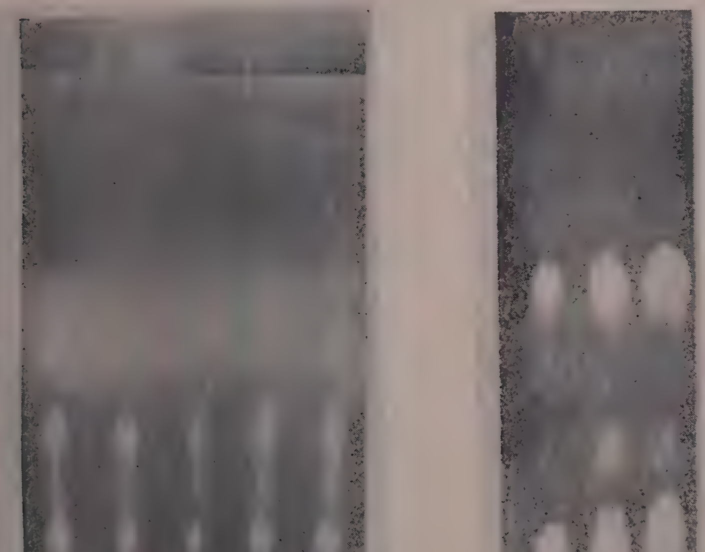

FIG. 2. Photograph of the chromatogram, run in B.F.W., of the oxidation of quinic acid after 25–30 hours (upper portion) and after 40–42 hours (see text).

Preparative scale oxidation of quinic acid. Early chromatograms were obtained using approximately 20 mg. quinic acid and 1 ml. of H_2O_2 refluxed at 110° C. for periods up to 3 hours. It was found unsatisfactory to increase this scale directly since the H_2O_2 was apparently fairly readily destroyed and the resultant water so diluted the reaction mixture that the rate of reaction was seriously reduced. It was therefore necessary to add H_2O_2 by stages. Nine grammes of quinic acid were refluxed with 25 ml. of H_2O_2 at 120° C. with the further addition of H_2O_2 from time to time to a total of 55 ml. Fig. 2 (upper part) shows paper chromatograms run in B.F.W. after 25–30 hours' reaction and after 40–42 hours (upper part). It will be seen that at 42 hours (the reaction was stopped after 44 hours) the reaction liquid contained chiefly two acids, citric acid and the unknown ('X') acid, with the latter predominating. It is interesting to note that during the oxidation the reaction liquid became and remained bright red so long as quinic acid was present; as soon as chromatograms showed this acid to have disappeared the reaction liquid became practically colourless. No explanation of this phenomenon has been found.

At the end of the oxidation period the liquid was boiled vigorously without the reflux condenser, to remove as much remaining H_2O_2 as possible, and then diluted to approximately 2 litres. The titratable acid present was 2·5 g. calculated as citric acid. The liquid was passed down a column containing 71 g. (air-dry weight) of deacidite E (70–100 mesh) at a rate of approximately 50 ml. per hour. After washing the column the acids were displaced by 0·1 N. HCl (60 ml. per hour), the displacement liquid being collected in several fractions. Citric and 'X' acid were present only in the last few fractions containing also traces of HCl. For further fractionation these fractions were combined and evaporated to dryness *in vacuo* at room temperature to remove HCl. The yellowish semi-solid residue weighed 225 mg. It was dissolved in 50 ml. of distilled water and the two acids present fractionated on a small column of Dowex 2 (2 g. dry weight). Much of the displacement liquid (by 0·1 N. HCl) from this column contained both citric and 'X' acids, but paper chromatograms showed that the last 8 ml. before the HCl broke through contained 'X' acid and this liquid was evaporated to dryness *in vacuo* to remove any traces of HCl. It was then taken up in a little warm water and shaken with charcoal. After filtration and evaporation of the filtrate again to dryness *in vacuo* the almost white solid was recrystallized. It had a melting-point (uncorrected) of 132° C. and formed a *p*-nitrobenzylbromide derivative having a melting-point of 84·5° C. On running chromatograms of the acid mixed with pure malonic acid (in both B.F.W. and E.Am.W.) a single spot only was obtained in each case. It was concluded, therefore, that 'X' acid was, in fact, malonic acid.

Oxidation of citric acid. Twenty milligrammes of citric acid were refluxed with 1 ml. of H_2O_2 under the same conditions as used in similar scale experiments with quinic acid. Chromatograms prepared in B.F.W. from spots of the reaction mixture were similar to those shown in Fig. 1, acids D to G. The eneral picture suggested that acids D to G (Fig. 1) represented stages in the xidation of citric acid and that none of these acids was involved in the oxidaion of quinic to citric acid (stage 1).

A chromatogram run in E.Am.W. of the oxidation mixture of citric acid fter $3\frac{1}{2}$ hours' refluxing is shown in Fig. 3, which also includes marker-spots of citric and malonic acid. Presumably acids 2 and 3 correspond as a pair to acids E and F on the B.F.W. chromatogram.

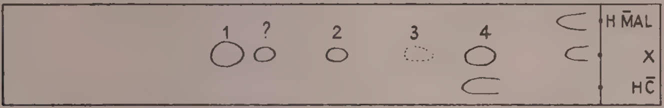

FIG. 3. Chromatogram, run in 'E.Am.W.', of the oxidation liquid of citric acid (X in the figure). Marker spots of citric (H$\bar{\text{C}}$) and malonic (H $\overline{\text{Mal}}$) acids are also shown (see text).

DISCUSSION

The R_F and R_M values of the seven acids represented by the spots in Fig. 1 are given in Table I. We have not found it possible to obtain exactly consistent values for the R_Fs of organic acids on different chromatograms unless such elaborate precautions are taken as to render the method tedious in routine work; indeed, provided marker-spots of known acids are run on chromatograms, this is no serious disadvantage. Nevertheless the R_M values in Table I are of interest since ΔR_M values in a series remain reasonably constant even when the absolute R_F values are varying to an appreciable amount. The significance of the approximately constant value of ΔR_M in the present series cannot be assessed so long as the identity of some of the members is unknown.

TABLE I

Acid	R_F value	R_M value	ΔR_M
A (quinic acid)	0·18	+0·66	
			0·16
B	0·24	+0·50	
			0·16
C	0·31	+0·34	
			0·15
D (citric acid)	0·40	+0·19	
			0·14
E	0·47	+0·052	
			0·15
F	0·56	−0·10	
			0·14
G (malonic acid)	0·63	−0·24	

Acids B and C remain unknown although one of them may be citricdialdehyde.

The identity of acids E and F is also conjectural, but it is probable (from e.g. Denigès Test—Denigès, 1902) that one is acetonedicarboxylic acid. Both have an ephemeral existence, F disappearing more rapidly than E.

The existence of acetonedicarboxylic acid as an intermediate in the oxidation of citric acid (and, consequently, of quinic acid) is corroborated by the universal appearance of acetone (detected as the 2:4-dinitrophenylhydrazone) in the oxidation products of both acids.

Malonic acid on oxidation breaks down to acetic acid and considerable amounts of acetic acid were found in the evaporates of the quinic and citric oxidation liquids. This breakdown to acetic acid is undoubtedly a contributing cause of the very low final yield of malonic acid and may account for malonic acid not being previously reported as an *in vitro* oxidation product of citric acid.

It has therefore been firmly established that peroxide oxidation of quinic acid yields citric and malonic acids. In addition there is strong presumptive evidence that acetonedicarboxylic acid is formed during the conversion of citric to malonic acid.

In an earlier paper (Hulme, 1951) the direct aromatization of quinic acid in plants, possibly via shikimic acid, to such benzenoid compounds as protocatechuic acid has been discussed. Although such reactions are known to occur in micro-organisms, their widespread occurrence in vegetative organs of the higher plants is not proved.

In the literature on plant biochemistry and physiology from the time of Gorter (1909) the widespread occurrence of the depside chlorogenic acid has been reported. Gorter showed 'chlorogenic acid' to be composed of one molecule each of caffeic acid and quinic acid. Recent work has shown the presence of chlorogenic acid-like compounds in various plant tissues (e.g. in the sweet potato by Rudkin and Nelson, 1947), but the results of the application of the modern techniques of partition chromatography suggest that 'chlorogenic acid' as used by some of the older workers is a general term for several closely allied depsides. Free quinic acid may, however, act as a source of citric (or even malonic) acid, and although Hall (1937) suggests that plants noted for their quinic acid content are not producers of citric acid, we have some evidence that citric acid appears in mature apples.

Mr. L. S. C. Wooltorton carried out most of the practical work involved in this paper.

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A Study of the Absorption and Utilization of Phosphate by Young Barley Plants

II. THE EFFECT OF PHOSPHATE STATUS AND ROOT METABOLISM ON THE DISTRIBUTION OF ABSORBED PHOSPHATE BETWEEN ROOTS AND SHOOTS

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SUMMARY

The effects of the phosphate status of young barley plants and of respiratory inhibitors on the distribution of phosphate absorbed during experimental periods lasting 24 hours has been studied.

The pretreatment of plants with 10 p.p.m. P increases the upward movement of phosphate when the plants are subsequently treated with 0·001 p.p.m. P. No such effect occurs when 10 p.p.m. P are supplied.

Sodium azide, 2:4-dinitrophenol, and diethyldithiocarbamate reduce the absorption of phosphate when the concentration of the external solution is 10 p.p.m. When, however, 0·001 p.p.m. P is provided, contrasting results are obtained, the most striking feature being that a considerable increase in the content of shoots may be induced. The extent of this effect is variable. A series of experiments with 10^{-3} M. azide indicates that the extent to which shoot content is increased depends on the extent to which phosphate is retained in the roots in the absence of the inhibitor.

These results suggest that the small amount of absorbed phosphate found in the shoots of plants supplied with low concentrations of phosphate is due to the metabolic retention of phosphate in the roots. It appears that two apparently distinct active processes in roots are subject to inhibition by the three inhibitors employed, namely metabolic retention and the transfer of ions across the root to the stele. The relative extent to which these processes are inhibited depends on the condition of the plants; metabolic retention may be reduced without comparable effects on absorption. It is considered that these results are incompatible with the hypothesis that absorption is directly mediated by the electron transfer in respiration. An alternative mechanism is discussed.

INTRODUCTION

In the preceding paper (Russell and Martin, 1952) it was shown that when barley seedlings which had been raised in the absence of external sources of phosphate were supplied with varying concentrations of labelled phosphate, an abnormally large proportion of the absorbed phosphate was retained in the roots of plants treated with the most dilute solutions (e.g. 0·001 p.p.m. P).

It was further shown that when plants which had been treated with such solutions were transferred to phosphate-free solutions, a larger proportion of

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he recently absorbed phosphate was lost to the outer medium than when igher concentrations of phosphate had been supplied. The retention of hosphate in the roots was shown to be directly attributable to the amount f phosphate entering the plants and not to an interaction between phosphate nd other ions simultaneously applied. The results could not be explained by he absorption of phosphate on inert surfaces in plants supplied with low oncentrations of that ion.

Two possible explanations of these effects appeared to merit consideration: i) That phosphate distribution is largely determined by simple concentration effects, upward movement being slight unless the amount of phosphate entering the roots significantly raises their phosphate content. (ii) That phosphate s actively retained in the roots by metabolic processes against upward translocation, the extent of retention being greatest when the phosphate level in certain phases is least.

To test these possibilities experiments of the same general type as those described in the preceding paper were undertaken, but with plants of varying initial phosphate status, and in the presence and absence of metabolic inhibitors. The cultural and analytical procedures employed have already been described (Russell and Martin, 1953). In discussing the results, the term 'transport index' is used, as in the previous paper, to denote the percentage of the phosphate absorbed during an experiment which was found in the shoots. Except where it is stated to the contrary, the plants received no phosphate from external sources prior to the experiments.

EXPERIMENTAL RESULTS

A. *The effect of phosphate status on the transport index*

In two experiments the effect of the phosphate status of plants on the subsequent absorption and distribution of phosphate was investigated by pretreating plants with unlabelled phosphate solutions containing 10 p.p.m. P for 24 hours. The control plants were supplied with phosphate-free solutions during this period. Changes in phosphate content per plant during the pretreatment period were determined by providing additional replicates with 10 p.p.m. labelled phosphate and sampling them at the commencement of the experiment.

Experiment H. Seedlings at the second-leaf stage were pretreated in the manner described and then transferred to labelled media containing 10 or 0·001 p.p.m. P for 24 hours. The pretreatments and treatments were combined factorially in sixfold replication. The experiment was carried out simultaneously with Expt. F (Russell and Martin, 1953), the controls being common to both experiments.

The results of Expt. H are shown in Table I and Fig. 1. Since it is known that the initial phosphate content per plant (i.e. that present in the seed) was 0·14 to 0·15 mg., it follows that the phosphate absorbed during the pretreatment period increased plant content by approximately 60 per cent. The absorption and distribution of phosphate was not significantly affected by pretreatment when the concentration of phosphate during the experimental period was 10 p.p.m. P. However, in plants supplied with 0·001 p.p.m. P, pretreatment caused a 30- to 40-fold increase in shoot content, from 0·0004 to 0·0149 μg. per plant. Root content was also increased, but the effect on total absorption was not significant.

Experiment J. In this experiment the treatments were the same as in Expt. H, but the plants employed were at the eighth-leaf stage. Early in their growth they had been provided with 0·15 mg. additional phosphate per plant. The results, shown in Table I and Fig. 1, contrast in many respects with those of Expt. H. Phosphate absorption during the pretreatment period was markedly greater and corresponded to approximately a fivefold increase in plant content. This undoubtedly reflected the lower phosphate status of the plants in Expt. J which resulted from their greater size (see Table I). In the 10 p.p.m. P treatment of this experiment, shoot content was significantly reduced by pretreatment with 10 p.p.m. P, while root content was significantly increased, whereas in Expt. H pretreatment had been without effect at this level of phosphate. In the 0·001 p.p.m. P treatment of Expt. J, however, pretreatment again increased shoot content and, although the effect was considerably less

TABLE I

Effect of pretreatment with phosphate (10 p.p.m. P) for 24 hours on the absorption and distribution of labelled phosphate in young barley plants. When a logarithmic transformation was necessary for statistical analysis the transformed values are shown in italics

Initial concentration of solution (p.p.m. P)		μg. P per plant			
Pretreatment	Treatment	Root	Shoot	Total	Transport index
		Experiment H (Mean dry weight per plant 0·11 g.)			
10	*	43·4	55·0	98·4	56·0
nil	10	39·9	51·9	91·8	55·8
10	10	42·0	52·5	94·5	55·5
	S.D.	n.s.	n.s.	n.s.	n.s.
nil	0·001	0·0647	0·0004	0·0651	0·63
			0·604		*0·799*
10	0·001	0·0649	0·0149	0·0798	18·80
			2·173		*2·274*
	S.D.	n.s.	*0·163*	n.s.	*0·152*
		Experiment J (Mean dry weight per plant: 0·92 g.)			
10	*	310	977	1287	76·0
nil	10	345	1291	1636	79·0
10	10	363	834	1197	69·5
	S.D.	n.s.	256	126	6·0
nil	0·001	0·442	0·150	0·592	25·4
10	0·001	0·153	0·412	0·565	72·9
	S.D.	0·048	0·042	n.s.	8·3

* Sampled at end of pretreatment period.

han in Expt. H, it was highly significant. The effects of pretreatment on the ate of absorption in the 0·001 p.p.m. P treatment of Expt. J could not be ssessed, since the culture medium was exhausted of phosphate to the extent f 95 per cent. This compared with 25 per cent. exhaustion in Expt. H and rovided further evidence of the greater absorption of the older plants.

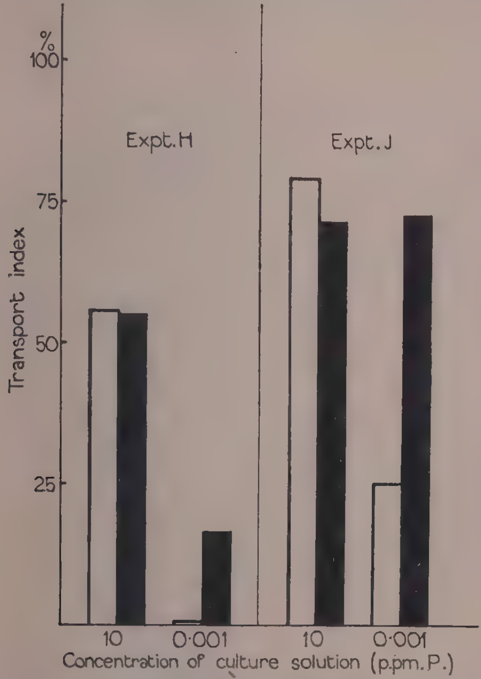


FIG. 1. Effects of pretreatment with phosphate on the transport indices of plants subsequently supplied with 10 and 0·001 p.p.m. P for 24 hours.
Shaded columns: plants pretreated with 10 p.p.m. P.
Unshaded columns: controls.

The feature of these results which is of particular relevance to the present investigation is that in both experiments, despite differences in the age and phosphate status of the plants, pretreatment with 10 p.p.m. P caused shoot content to be increased when the plants were subsequently supplied with 0·001 p.p.m. P. This is indicative that the transfer of phosphate from the roots of plants to shoots under such conditions is not determined by simple concentration effects. Had the extent to which absorption raised the phosphate content of the roots determined upward translocation, this process would have been reduced by pretreatment with phosphate. It is noteworthy that the amount of labelled phosphate found in the shoots was increased by pretreatment with phosphate only when the concentration of phosphate provided during the experimental period was low, that is to say under the condition which in the absence of pretreatment would have caused considerable phosphate retention in the roots. This situation is compatible with the interpretation that certain processes in the root retain phosphate against upward movement when both the phosphate status of the plants and the amount of phosphate absorbed is low. Pretreatment with phosphate appears to have satisfied partially the requirements of these processes and so permitted greater upward movement of the phosphate absorbed during the subsequent experimental period.

B. *The effect of metabolic inhibitors on the distribution of absorbed phosphate between roots and shoots*

The foregoing results suggest that the retention of phosphate in the roots of plants supplied with low concentrations of phosphate is due to metabolic processes. It therefore appeared appropriate to examine the effects of enzyme inhibitors on the distribution of phosphate. The aeration system employed in the culture vessels made it impossible to use gaseous inhibitors or substances which are readily removed by an air-stream. Carbon monoxide and cyanide could therefore not be employed. Sodium azide was chosen as the most suitable substance for a preliminary investigation as it is known to inhibit both respiration and salt uptake under normal conditions and the volatility of HN_3 did not seem significantly to affect the results. 2:4-dinitrophenol was also employed. There is strong evidence (James and Garton, 1952) that respiration in barley roots is largely mediated by copper systems, presumably ascorbic acid oxidase: the effect of diethyldithiocarbamate, which under appropriate conditions can be employed as a specific inhibitor for such systems (King, 1939; James and Garton, loc. cit.), was therefore examined.

1. *Sodium azide*

Experiment K. Plants at the second-leaf stage were treated for 24 hours with 10 and 0·001 p.p.m. P combined factorially with 10^{-3}, 10^{-4}, and 10^{-5} M. sodium azide. Control treatments at both levels of phosphate were included. In additional treatments 10^{-4} and 10^{-5} M. dinitrophenol were employed. Replication was fivefold. The detailed results for the azide treatments are shown in Table II. In Fig. 2 the principal effects are represented graphically.

When plants were supplied with 0·001 p.p.m. P, the shoot content was greatly increased by azide, the effect being maximal in the 10^{-3} M. azide treatment; root content was not significantly affected by azide and the overall effect on absorption was not significant. By contrast, when plants were supplied with 10 p.p.m. P, phosphate absorption was markedly reduced by all concentrations of azide, shoot content being depressed more than root content. Thus, whereas the transport index was increased by azide in plants supplied with 0·001 p.p.m. P, the reverse effect occurred when 10 p.p.m. P were employed.

Experiment L. In this experiment the 10^{-3} and 10^{-5} M. azide treatments of Expt. K were repeated. The experimental procedures, however, differed in a umber of respects. Three levels of phosphate, 10, 0·1, and 0·001 p.p.m. P, ere employed and following the 24-hour treatment (period I), plants were aintained in phosphate-free media for 24 hours (period II). During this eriod a series of replicates which had previously received no azide were 'post-reated' with 10^{-3} M. azide. This experiment was carried out simultaneously ith Expt. C (Russell and Martin, 1953), the controls being common to both xperiments.

TABLE II

xperiment K: Effect of sodium azide on the absorption and distribution of hosphate in young barley plants. (Five replicates.) When a logarithmic trans-ormation was necessary for statistical analysis the transformed values are shown in italics

Initial concentration of culture solution		μg. P. per plant			
p.p.m. P	Azide (Molar)	Root	Shoot	Total	Transport index
10	nil	53·3	108·4 *2·035*	161·7 *2·209*	66·8
	10^{-5}	40·0	44·0 *1·644*	84·0 *1·924*	52·5
	10^{-4}	40·0	10·8 *1·033*	39·3 *1·594*	26·9
	10^{-3}	18·8	2·66 *0·425*	21·5 *1·332*	12·8
	S.D.	9·1	*0·154*	*0·129*	6·7
0·001	nil	0·0984	0·0020 *0·301*	0·1004	1·80 *0·255*
	10^{-5}	0·1152	0·008 *0·909*	0·1233	6·64 *0·822*
	10^{-4}	0·1160	0·0100 *1·000*	0·1260	8·27 *0·918*
	10^{-3}	0·0983	0·0134 *1·127*	0·1118	12·04 *1·081*
	S.D.	n.s.	*0·218*	n.s.	*0·186*

Mean dry weight per plant: 0·076 g.

The shoot content of plants treated with 0·001 p.p.m. P was again increased by azide (Table III); root content was, however, reduced. In plants supplied with 10 p.p.m. P, absorption was markedly reduced as in the previous experiment. At the 0·1 p.p.m. P level the effect of azide was intermediate between those shown at the extreme phosphate levels.

When 10^{-3} M. azide was applied in period II no significant effects on the distribution of phosphate between roots and shoots was observed. In particular, it is to be noted that the considerable increase in shoot content which was induced by 10^{-3} M. azide when applied simultaneously with 0·001 p.p.m. P was not reproduced. Thus it appears that the inhibitor does not increase the upward movement of phosphate previously present in the plant. This effect has been confirmed in other experiments not here reported.

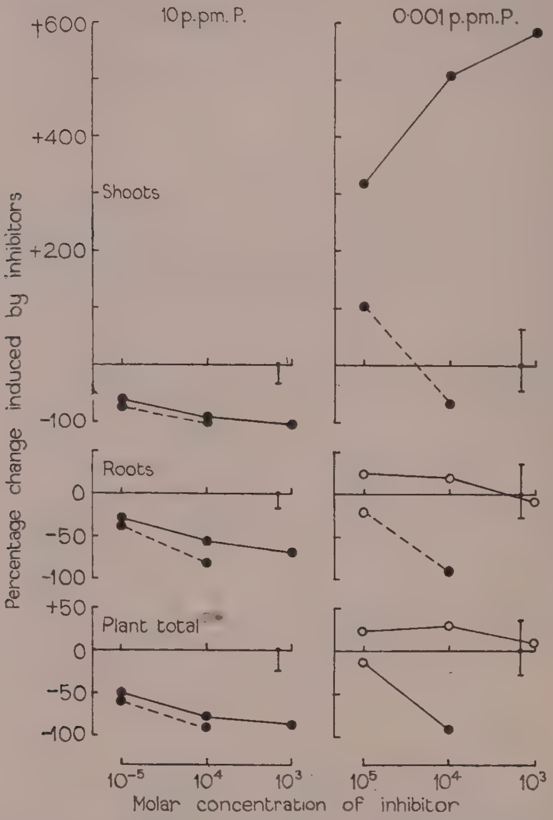


FIG. 2. *Experiment K*: Changes in the phosphate content of young barley plants induced by sodium azide (continuous lines) and 2:4-dinitrophenol (broken lines). Left: plants supplied with 10 p.p.m. P. Right: plants supplied with 0·001 p.p.m. P. Values are expressed as percentages of controls. Significant differences from the control treatments are shown and significant effects are indicated by solid circles.

From the viewpoint of elucidating the factors which induce the retention of absorbed phosphate in plants, the increased translocation to the shoots which occurred when sodium azide and 0·001 p.p.m. P were simultaneously applied appeared of particular interest. Only this concentration of phosphate was therefore used in subsequent experiments with azide.

Experiment M. The second leaves of the seedlings used in this experiment were only partially expanded, and the plants were barely a third of the size of those in Expts. K and L. The following concentrations of azide were

TABLE III

xperiment L: Effect of sodium azide on the absorption and distribution of phos- ate in young barley plants, treated for 24 hours with labelled phosphate eriod I) and subsequently for 24 hours in a phosphate-free solution (period II). ive replicates.) When a logarithmic transformation was necessary for statistical analysis the transformed values are shown in italics

Initial concentration of culture solution						
p.p.m. P (Period I only)	Azide (Molar)		µg. P per plant			Transport index
	Period I	Period II	Root	Shoot	Total	
10·0	nil	nil	112·4	168·8 *2·227*	281·2 *2·449*	59·2
	10^{-5}	nil	121·1	110·6 *2·044*	231·7 *2·365*	47·5
	10^{-3}	nil	64·1	7·50 *0·848*	71·5 *1·854*	10·4
	nil	10^{-3}	139·8	136·7 *2·136*	276·5 *2·442*	49·1
	S.D.	—	19·3	*0·176*	*0·089*	8·6
0·1	nil	nil	6·87	4·49 *0·652*	11·36	39·5
	10^{-5}	nil	6·09	4·99 *0·698*	11·08	45·1
	10^{-3}	nil	2·80	1·19 *0·075*	3·99	28·8
	nil	10^{-3}	4·50	4·76 *0·678*	9·26	51·5
	S.D.	—	0·73	*0·070*	1·09	4·8
0·001	nil	nil	0·0653	0·0033 *0·519*	0·0686	4·73 *0·675*
	10^{-5}	nil	0·0548	0·0071 *0·851*	0·0619	11·60 *1·075*
	10^{-3}	nil	0·0521	0·0175 *1·243*	0·0696	24·47 *1·389*
	nil	10^{-3}	0·0597	0·0044 *0·644*	0·0641	7·13 *0·853*
	S.D.	—	0·0086	*0·165*	n.s.	*0·155*

Mean dry weight per plant: 0·077 g.

employed: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} M. together with controls. In additional treatments 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M. dinitrophenol were included. Replication was fivefold. The effects of azide are shown in Table IV, and in Fig. 3 the principal results obtained with both inhibitors are represented graphically. Shoot content was increased, though not to a significant extent, by 10^{-5} and 10^{-4} M. azide, and reduced by higher concentrations. Total absorption was unaffected by 10^{-5} and 10^{-4} M. azide, but higher concentrations caused a very marked decrease. The effects on the transport index were

TABLE IV

Experiment M: Effect of sodium azide on the absorption and distribution of phosphate in young barley plants. Initial content of culture medium, 0·001 p.p.m. P. (Five replicates.) When a logarithmic transformation was necessary for statistical analysis the transformed values are shown in italics

Molar concentration of azide	μg P per plant			Transport index
	Root	Shoot	Total	
nil	0·02344	0·00526	0·02870	18·17
	2·370	*2·721*	*2·458*	
10^{-5}	0·01980	0·00495	0·02475	20·28
	2·297	*2·695*	*2·394*	
10^{-4}	0·02284	0·00475	0·02759	16·58
	2·358	*2·677*	*2·441*	
10^{-3}	0·01621	0·00092	0·01713	5·72
	2·210	*1·964*	*2·234*	
10^{-2}	0·00102	0·00068	0·00170	40·60
	1·009	*1·833*	*1·230*	
10^{-1}	0·00071	0·00004	0·00075	6·28
	0·851	*0·602*	*0·875*	
S.D.	*0·189*	*0·269*	*0·182*	7·01

Mean dry weight per plant: 0·025 g.

complex; in the 10^{-5} M. treatment it exceeded the control value, though not to a significant extent; when the concentration of azide was increased to 10^{-3} M. a significant decrease in the index occurred; in 10^{-2} M. treatment the index was over twice the control value, while at the highest concentration of azide (10^{-1} M.) it was again low. The plant content data show that the chief difference between the 10^{-2} and 10^{-3} M. treatments was a much lower value for roots in the former. This suggests that the high transport index in the 10^{-2} M. treatment may have been due to phosphate loss from roots following irreversible injury in the later stages of the experiment. Loss of electrolytes has been observed by other investigators in these circumstances (Stenlid, 1948).

The results of Expt. M contrast with those of Expts. K and L in that no increase in shoot content was induced by 10^{-3} M. azide when applied with 0·001 p.p.m. P. Since the principal differences between the experiments was that much younger plants were used in Expt. M., it was decided to investigate the effects of plant age on the response to azide.

Experiment N. Four batches of seedlings of which the 1st, 2nd, 3rd, and 4th leaf had emerged were treated for 24 hours with 10^{-5} and 10^{-3} M. azide together with controls. Replication was fourfold. The results (Table V) show that in the youngest plants shoot content was reduced at both concentrations of azide: when, however, the plants were at the 2nd-, 3rd-, and 4th-leaf stage, the content of shoots was increased by azide, the extent of the effect increasing with the age of the plants. Total plant content was decreased by 10^{-3} M. azide in the youngest plants and significantly increased in the plants at the 3rd- and 4th-leaf stage.

This experiment therefore supported the suggestion made on the basis of te results of Expt. M that shoot content was not increased by azide in young pants to the same extent as in older ones. It will be seen from Tables II to V tat the control plants at the 1st-leaf stage (Expts. M and N) contrasted aso with plants at a later stage of development by showing markedly higher tansport indices.

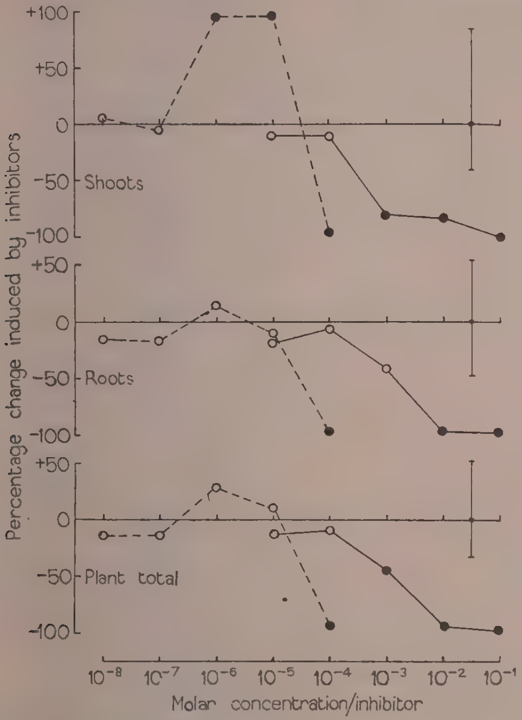


FIG. 3. *Experiment M*: Changes in the phosphate content of young barley plants induced by sodium azide (continuous lines) and 2 : 4-dinitrophenol (broken lines). Plants supplied with 0·001 p.p.m. P. Values are expressed as percentages of controls. Significant differences for the control treatments are shown and significant effects are indicated by solid circles.

The relationship between plant dry weight and the percentage increase in hoot content induced by azide has been examined for all experiments in vhich plants were sampled after treatment with azide for 24 hours (Fig. 4). The 10^{-3} M. azide treatment has been selected for this purpose since the greatest effects were induced by this concentration. A highly significant posiive linear regression of the percentage increase in shoot content on plant dry weight has been shown, the regression equation being

$$y = (0{\cdot}102 \pm 0{\cdot}021)x + 21{\cdot}4.$$

The possible significance of this relationship is discussed in a later section.

TABLE V

Experiment N: Effect of sodium azide on the absorption and distribution of phosphate in young barley plants of different ages. When a logarithmic transformatio was necessary for statistical analysis the transformed values are shown in itali

Youngest leaf fully emerged	Dry weight g. plant	Molar concentration azide	µg. P per plant Root	µg. P per plant Shoot	µg. P per plant Total	Transport index
1	0·0382	nil	0·0419	0·0051	0·0470	10·9
		10^{-5}	0·0393	0·0039	0·0432	9·0
		10^{-3}	0·0342	0·0017	0·0359	4·7
		S.D.	n.s.	0·0017	0·0097	3·0
2	0·0558	nil	0·0329	0·00023 *0·362*	0·0331	0·62 *0·792*
		10^{-5}	0·0246	0·00047 *0·672*	0·0251	1·73 *1·238*
		10^{-3}	0·0209	0·00132 *1·121*	0·0221	6·16 *1·790*
		S.D.	0·0107	*0·328*	0·0111	*0·180*
3	0·0979	nil	0·0734	0·0020 *0·301*	0·0754	2·7
		10^{-5}	0·0734	0·0028 *0·447*	0·0762	3·5
		10^{-3}	0·0849	0·0141 *1·149*	0·0990	14·2
		S.D.	0·0112	*0·263*	0·0136	2·2
4	0·145	nil	0·0137	0·00016 *0·204*	0·0139	1·18 *0·072*
		10^{-5}	0·0146	0·00022 *0·342*	0·0148	1·46 *0·164*
		10^{-3}	0·0159	0·00163 *1·212*	0·0175	9·32 *0·969*
		S.D.	0·0011	*0·327*	0·0011	*0·308*

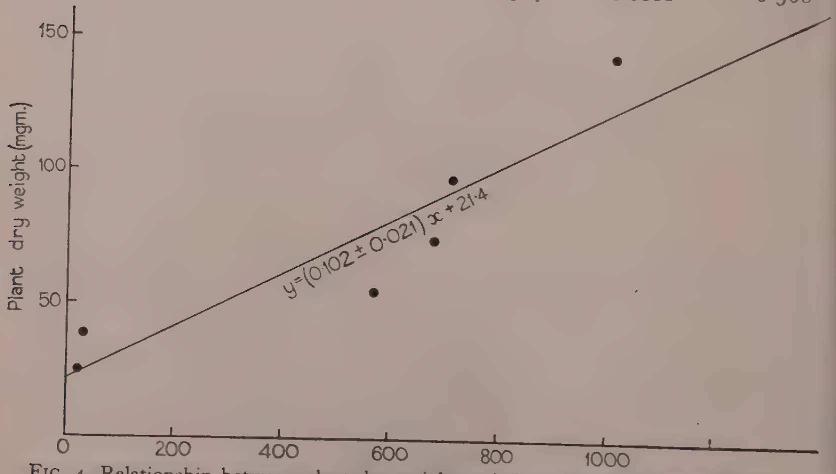


FIG. 4. Relationship between plant dry weight and the percentage change in shoot content induced by 10^{-3} M. azide in experiments of 24 hours' duration.

. *Other inhibitors.*

2:4-dinitrophenol. In two experiments already described, treatments with initrophenol were included, namely: Expt. K: 10^{-4}, 10^{-5} M.; Expt. M: 10^{-4}, 0^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M. together with controls. One further experiment (Expt. O) was undertaken in which the treatments of Expt. K were repeated on plants of similar age. The results for the three experiments are shown in Fig. 5. When 10 p.p.m. P were supplied, absorption was considerably reduced and the content of roots was depressed less than that of shoots. When, however, the concentration of phosphate was 0·001 p.p.m. P, shoot content was

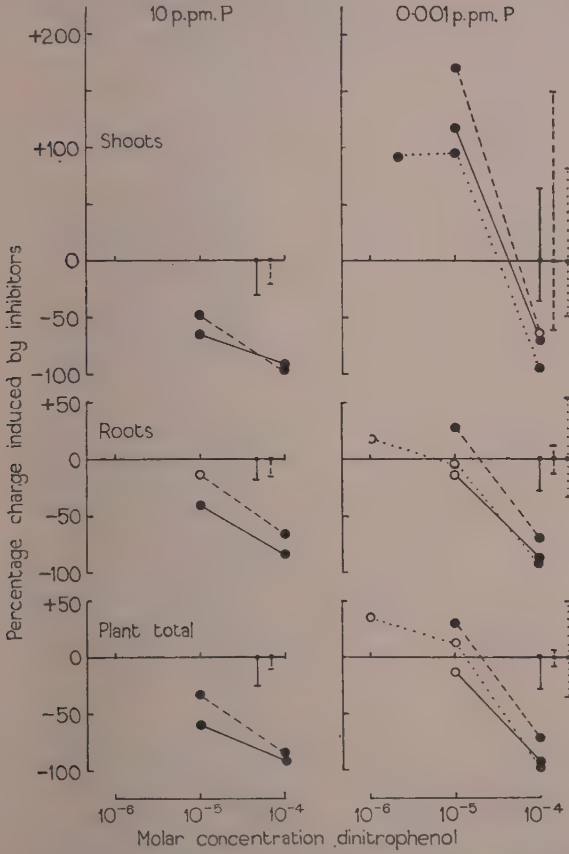


FIG. 5. Changes in phosphate content of young barley plants induced by 2:4-dinitrophenol. Left: plants supplied with 10 p.p.m. P. Right: plants supplied with 0·001 p.p.m. P. Values are expressed as percentages of the control treatment. Expt. K, continuous line; Expt. M, dotted line; Expt. O, broken line. Values are expressed as percentage of controls. Statistically significant effects are indicated by solid circles.

increased. There is thus a general similarity between the effects of dinitrophenol and those of azide. However, the relative effect of the two substances was variable. In Expt. K (Fig. 2) the shoot content of plants supplied with 0·001 p.p.m. P was increased by 10^{-3} M. azide to a considerably greater extent than by 10^{-5} or 10^{-6} M. dinitrophenol. In Expt. M (Fig. 3) the relative extent of the effects of the two inhibitors was reversed. This suggests that the extent to which dinitrophenol affects shoot content cannot be correlated with plant dry weight in the manner established for azide.

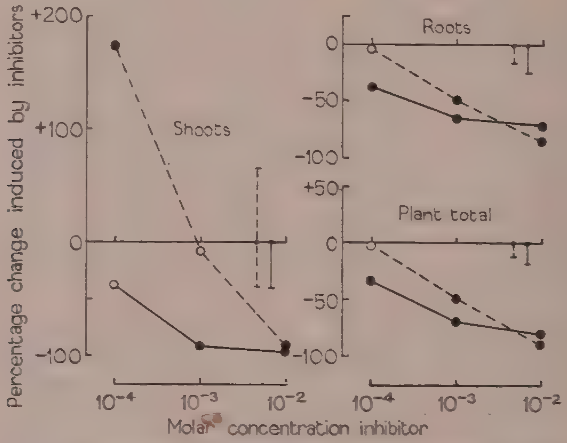


FIG. 6. *Experiment P*: Changes in phosphate content of young barley plants induced by diethyldithiocarbamate. Solid line: plants supplied with 10 p.p.m. P. Broken line: plants supplied with 0·001 p.p.m. P. Significant differences from the control treatments are shown and significant effects are indicated by solid circles.

Diethyldithiocarbamate. In Expt. P plants at the second-leaf stage were treated for 24 hours with the following concentrations of diethyldithiocarbamate and phosphate combined factorially: 10^{-2}, 10^{-3}, 10^{-4} M. diethyldithiocarbamate and control; 10 and 0·001 p.p.m. P. Replication was fivefold. The results are set out in Fig. 6. The general pattern of effects again resembled that established for azide.

DISCUSSION

The effect of enzyme inhibitors and phosphate status on phosphate distribution

In the experiments with sodium azide and other inhibitors here described, two types of effect were observed, namely: (i) the absorption of phosphate was either increased or unaffected, while shoot content was markedly increased by intermediate concentrations of inhibitor; (ii) absorption was reduced, the effect on shoot content being greater than that on root content. That respiratory inhibitors reduce absorption is in agreement with the findings of many

vestigators; the novel feature of the present results is the apparent stimula-
on of translocation to the shoots induced by such substances. This effect was
rought about by 10^{-3} M. azide when the transport index of the controls was
w, i.e. 5 per cent. or less, as was the case in the majority of experiments in
hich 0·001 p.p.m. P was employed. By contrast absorption, and especially the
ontent of shoots, was decreased in all cases when the transport index of the
ontrols was 10 per cent. or more, i.e. in all experiments when plants were
pplied with 10 p.p.m. P or when very young plants were supplied with
·001 p.p.m. P (Expts. M and N). The nature of the effect of azide on the
ovement of phosphate to shoots thus appears to be dependent primarily on
e extent of phosphate retention in the roots of untreated plants, and is only
directly related to the concentration of phosphate in the external solution.
t was shown in those experiments in which plants were pretreated with
hosphate (Table I) that the proportion of the absorbed phosphate retained
n the roots of plants supplied with 0·001 p.p.m. P was greatest when the
nitial phosphate status of the plants was low. Thus, if the extent of phosphate
etention in the roots of untreated plants determines the extent to which in-
ibitors increase shoot content, this effect should be greatest in plants of low
hosphate status. It is therefore pertinent to inquire whether the variable
ffects of inhibitors on the shoot content of plants supplied with 0·001 p.p.m.
P is correlated with the concentration of phosphate within them. Azide is the
nly inhibitor for which the data are adequate for such an examination.

It has been shown that when plants were supplied with 0·001 p.p.m. P, the percentage increase in shoot content induced by 10^{-3} M. azide was closely correlated with plant dry weight (see Fig. 4). Since all the plants had been grown in phosphate-free solutions, and negligible quantities of seed-borne phosphate were lost by outward diffusion during their growth (Russell and Martin, 1953), the mean concentration of phosphate at the commencement of the experiments was proportional to plant dry weight. Hence Fig. 4 indicates that a negative relationship existed between the mean initial phosphate status of the plants and the percentage change in shoot content induced by azide. It must be borne in mind that the different batches of plants for which data are summarized in Fig. 4 differed not only in dry weight and in mean initial phosphate content. The environment of the greenhouse in which the experiments were carried out could not be maintained constant and differences in growth rate consequently occurred between different batches of plants. Moreover, with increasing age, the pattern of growth changed; a progressive increase in the ratio of root dry matter to shoot dry matter was observed. Both these circumstances may be expected to have caused differences not only in phosphate utilization but also in the pattern of enzyme activity. A close relationship between the percentage increase in shoot content induced by azide and the mean initial phosphate content of the plants would consequently not be expected if the effect of the inhibitor was due to its interference with metabolic processes which varied independently of the weight and phosphate status of the plants. Thus, unless the relationship shown in Fig. 4 is fortuitous, it is most simply interpreted as indicating that, in the absence of inhibitors, the extent of phosphate retention by certain mechanisms in the roots is determined primarily by the mean concentration of phosphate within them, and that azide directly inhibits such retention, thus causing a larger proportion of the absorbed phosphate to be secreted into the xylem.

2:4-dinitrophenol induced effects generally similar to those of sodium azide. The relative extent to which the two inhibitors affected the shoot content of plants supplied with 0·001 p.p.m. P, however, varied between experiments; thus their modes of action cannot have been identical. Diethyldithiocarbamate also caused comparable effects.

The method employed in these experiments precluded the simultaneous observation of the effects of inhibitors on respiration and on the utilization of phosphate. The inhibition of respiration by azide is, however, well established. It has been shown by James (private communication) that 10^{-3} M. azide reduces by over 90 per cent. the respiration of root-tips detached from plants similar to those here used, and that diethyldithiocarbamate likewise inhibits their respiration (James and Garton, 1952). Concentrations of dinitrophenol which reduce salt uptake have been shown to increase respiration (Robertson, Wilkins, and Weeks, 1951), but there is evidence that this substance inhibits the mechanism of energy transfer from respiration (Loomis and Lipmann, 1948) and the rise in respiration rate is probably caused by uncoupling of phosphorylation. Thus, although no quantitative relationship between the effects of the inhibitors on metabolism and on the distribution of phosphate can be suggested, it may be inferred that the increased movement of phosphate to the shoots which was induced by these substances was associated with the inhibition of either respiration or energy transfer.

The results of the present experiments together with those obtained when plants were pretreated with phosphate therefore provide strong evidence that the retention of phosphate by metabolic processes in the roots limits translocation to the shoots when the amount of phosphate entering the plant is small, especially when the plants are of low initial phosphate status. Phosphate held in plant roots in this manner will be referred to as 'metabolically retained' in the subsequent discussion.

In addition to inhibiting metabolic retention the three inhibitors have been shown to depress the total amount of phosphate absorbed, the effect being particularly great when a high external supply of phosphate (10 p.p.m. P) is provided. Two apparently distinct mechanisms were therefore subject to inhibition, namely that responsible for the normal entry of ions and that responsible for their retention in roots. Dependent on the metabolic condition of the plants, either of these effects may be so great that it masks the other.

The mechanism of the retention of phosphate in plant roots

That metabolic processes in the root may retain ions against upward translocation is well recognized. van Andel, Arisz, and Helder (1950) have found it convenient to distinguish three types of metabolic process in which osphate entering roots may be utilized, namely (i) accumulation in the cell sp, (ii) metabolic utilization in the zone of growth, (iii) radial transport and cretion to the xylem. Any increase in the relative extent of the first two prosses would clearly reduce the third. Evidence of the metabolic retention of hosphate against upward movement has also been provided by investigaon of the effects of radiation from absorbed P^{32} on intact barley plants. creased upward translocation has been found to be one of the most sensitive dices of radiation injury (Russell and Martin, 1952). This effect is attriuted to the irradiation of root-tips by the accumulated P^{32}.

A question of obvious interest is whether metabolic retention represents the owing down of the normal process which transfers phosphate to the stele, r its incorporation in other complexes. The fact that respiratory inhibitors educed metabolic retention suggests that the latter is the more reasonable nterpretation. Further evidence in this direction is provided by the fact that zide accelerated the upward movement of phosphate to the shoots only when pplied simultaneously with phosphate; phosphate which had already been etabolically retained was not subject to this effect (Table III). It may be ssumed therefore that the metabolic retention of phosphate is due to its diverion into channels distinct from those through which it passes in its normal assage across the root. It remains to be considered whether accumulation ccurs in the cell sap, or whether anabolic utilization provides the most likely xplanation.

In the preceding paper (Russell and Martin, 1953) it was shown that when plants were transferred from labelled phosphate to phosphate-free solutions, the proportion of the labelled phosphate which was released into the outer medium was greatest when plants had been treated with low concentrations of phosphate, i.e. when a relatively large fraction of the phosphate absorbed during the experimental period was retained in the roots. Metabolically retained phosphate therefore appears to be particularly subject to loss by outward diffusion. It is, however, not known whether organic complexes, or inorganic phosphate formed by their breakdown, are lost in this manner. The seemingly paradoxical situation that plants supplied with low concentrations of phosphate lose recently absorbed phosphate particularly readily to the outer medium is suggestive that metabolic retention occurs in the cytoplasm and not in cell vacuoles, from which loss would be expected to be slower.

The present results provide no direct evidence as to the nature of the complexes in which phosphate is metabolically retained. There are indications that the process occurs mainly in apical meristems; studies of phosphate distribution in root segments show that the concentration gradient from the root-tips to the older tissues is greatest when the external concentration of phosphate is low (Table VI). Both for this reason and because the roots of phosphate deficient plants are abnormally well developed relative to their shoots (e.g. Williams, 1948; Humphries, 1951) it appears not unlikely that metabolically retained phosphate is utilized in processes associated with growth. The fact that phosphate retention in roots is readily reduced by ionizing

TABLE VI

Concentration of labelled phosphate in the roots of young barley plants treated for 24 hours with labelled phosphate. Subsequent to treatment the roots were divided into segments 5 mm. long. 5% fiducial limits are shown.

	Initial content of culture medium (p.p.m. P)					
	10		0·1		0·001	
Concentration in apical segment as percentage of initial content of culture medium	65·8±7·7		2955±424		1521±424	
Concentration in proximal segments as percentage of concentration in apical segment:						
5–10 mm. from apex	56·0	±8·3	31·0	±2·7	33·6	±3·8
10–15 mm. " "	61·4		19·2		22·1	
15–20 mm. " "	50·8		17·5		20·9	

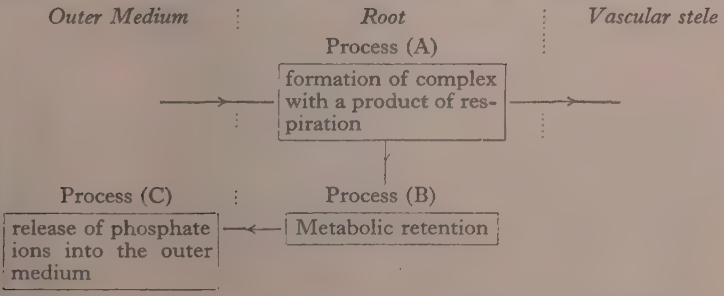
radiation conforms with this interpretation since meristematic tissues are particularly sensitive to radiation injury and the synthesis of nucleic acid is more readily retarded by radiation than protein synthesis (Abrams, 1951).

The energy relations of phosphate absorption and utilization

Lundegårdh (1950) and Robertson and Wilkins (1948) have envisaged the absorption of salts as being dependent on 'anionic respiration'; the electron transfer in the cytochrome oxidase system has been visualized as directly mediating the movement of anions both across the cytoplasm of single cells and across the root to the vascular cylinder. Robertson, Wilkins, and Weeks (1951) have lately found it necessary to conceive the existence of additional mechanisms, but have apparently not abandoned the concept of 'anionic respiration'. If salt absorption is correctly explained by these workers, the inhibition of the fraction of respiration associated with this process must in all circumstances immediately inhibit salt uptake. Moreover, since 'salt respiration' is more sensitive to inhibition than 'ground respiration' or the 'third respiration' of root-tips (Lundegårdh, loc. cit.) it would seem that salt uptake must be at least as sensitive to inhibition by respiratory inhibitors as any other metabolic process. The present results show that this is not necessarily the case. It has been possible to distinguish between the effects of respiratory inhibitors in *two* active processes, namely metabolic retention of phosphate in the roots and the transfer of phosphate across the root and its subsequent release to the stele. Inhibitors may reduce metabolic retention and accelerate upward transport when the condition of the plants is such that a large fraction of the absorbed phosphate would otherwise be metabolically retained in the roots. This effect cannot be dismissed as an abnormality occasioned by the very low concentrations of salt provided (i.e. 0·001 p.p.m. P) since it was not always induced by low external concentration (*vide* Expts. M and N). It appears therefore that the present results are incompatible with the mechanism of absorption envisaged by Lundegårdh and Robertson. Alternative mechanisms must therefore be considered.

The existence of metabolically produced carriers which combine with ions at or near the cell surface and mediate their subsequent accumulation or ecretion has been postulated by many workers (e.g. Wohl and James, 1942; Steward and Street, 1947; Jacobsen, Overstreet, King, and Handley, 1950). The suggested mechanisms contrast in several respects, but they share the common postulate that there is an *indirect* connexion between respiration and salt uptake. This implies that at any instant an actively absorbing tissue must possess some capacity for salt accumulation dependent on a product of *prior* respiration. This characteristic is shown also by the mechanism of secretion dependent on phosphate esters which Davies and Ogston (1950) have developed. It is of interest therefore to consider whether the present results are compatible with the hypothesis that salt accumulation is *indirectly* dependent on respiration.

It is simplest to visualize three processes, though each in all probability embraces a number of stages: *Process* (*A*) represents the formation at, or near, the cell surface of a complex between the entering ion and a product of respiration. This process is envisaged as resulting in the ultimate release of the ion into the stele unless the ion is diverted into *Process* (*B*), the mechanism of metabolic retention. *Process* (*C*) results in the release into the outer medium of ions which had previously been utilized in Process (B) (*vide* p. 151 above). Since it is postulated that the root possessed a certain capacity to accomplish Process (A) in consequence of *prior* respiration, it follows that if the number of ions available for absorption does not exceed this capacity the inhibition of


Outer Medium
Root
Vascular stele
Process (A)
formation of complex with a product of respiration
Process (C)
release of phosphate ions into the outer medium
Process (B)
Metabolic retention

respiration will not inhibit Process (A). By contrast, if the number of ions available for absorption is greatly in excess of the instantaneous capacity of the root for absorption, the rate of Process (A) will be directly dependent on respiration occurring simultaneously with salt absorption. Furthermore, if Process (B) is inhibited to a greater extent than Process (A), the rate of release of ions into the vascular stele will be *increased*. Moreover, the inhibition of Process (B) may cause an apparent increase in total absorption in consequence of the reduced extent of Process (C).

The results obtained in the present experiments could be explained on this basis. When the external concentration of phosphate was high (10 p.p.m. P) absorption was greatly reduced, so also was the proportion of the absorbed phosphate translocated to the shoot; under these circumstances the rate of Process (A) may be regarded as having been closely related to the rate of simultaneous respiration. When the external concentration of phosphate was low (0·001 p.p.m. P) and the extent of metabolic retention in the plants not treated with inhibitors was also low, absorption and translocation to the shoot were again reduced by inhibitors, though to a much smaller extent, as would be expected if the products of prior respiration were able to mediate the absorption of a significant proportion of the ions in the medium. When, however, the external concentration of phosphate was low (0·001 p.p.m. P) and the extent of metabolic retention in the plants not treated with inhibitors was high, upward movement to the shoot was increased by inhibitors, and in some cases the total amount of phosphate absorbed was increased also; the increased shoot content may be interpreted as indicating that the extent of inhibition of Process (B) was sufficiently greater than the extent of inhibition of Process (A) to cause a greater rate of release of ions into the vascular stele. The increase in total plant content induced by inhibitors in some experiments suggests that phosphate loss through Process (C) was reduced to a greater extent than primary absorption (Process A).

In the foregoing discussion the effects of the supply of phosphate and of the metabolic activity of the roots in the partition of phosphate between Processes (A) and (B) have alone been considered. It is, however, apparent from the observation of many investigators that metabolic processes in the shoot also influence phosphate utilization in the roots of intact plants (Steward, Prevot, and Harrison, 1942; Alberda, 1948; Helder, 1951). The examination of this question is, however, beyond the scope of the present investigation.

The speculative nature of the interpretation here advanced is sufficiently obvious to require no emphasis. The conclusion of a number of workers that phosphate enters cells as organic esters (e.g. Wildman, Campbell, and Bonner, 1949) would, however, appear to accord with the view that absorption is mediated by some carrier mechanism. It may be noted also that the observations of Woodford and Gregory (1948) that a reduction of oxygen tension could increase phosphate absorption may perhaps be interpreted on the grounds that the reduction in metabolic utilization may have reduced phosphate loss through the postulated Process (C).

It would clearly be unwarranted to assume that any conclusions reached with regard to the absorption of phosphate are applicable to other ions also. However, it is of interest that no data so far available are incompatible with the theory that absorption is dependent on respiration through an intermediate as yet unidentified; evidence frequently cited in support of the concept of 'anionic respiration' is equally compatible with this interpretation. The induction of 'salt respiration' in starved tissues when external supplies of salt are provided could be explained on the grounds that the rate of formation of the respiratory intermediate is conditioned by its rate of utilization.

The fact that salt accumulation and respiration are both reversibly inhibited by carbon monoxide is compatible equally with Lundegårdh's views and with the postulate that cytochrome can bring about the synthesis of the carrier molecule. The observations of Robertson and Wilkins (1948) that the rate of accumulation of anions does not exceed the theoretical expectation on the basis that one electron leaving respiration mediates the entry of one ion, could be interpreted as indicating that the transfer of one electron is required for the production of the intermediate which mediates the absorption of one ion. The difficulties Robertson, Wilkins, and Weeks (1951) have experienced in reconciling the fact that dinitrophenol may inhibit salt uptake without inhibiting respiration can be explained by the postulate that this substance inhibits the formation of the intermediate by interference with energy transfer. This interpretation is at least as simple as their explanation involving mitochondria.

In view of the complexity of the interacting processes which occur simultaneously within cells, it would appear unrealistic to advance detailed biochemical interpretations solely on the basis of the effects on respiration and salt absorption induced in tissue slices or intact plants by changes in the external concentration of salts, or by the use of enzyme inhibitors. It may be suggested that the value of such work lies principally in that it enables postulates to be classified into those which merit further examination and those which are untenable. The present results indicate that the intimate relation between respiration and the absorption of ions which Lundegårdh postulated is inapplicable to the absorption of phosphate. It is of interest that Sutcliffe (1952), who examined the salt respiration of storage tissue supplied with potassium salts, reached a similar conclusion. The elucidation of the mechanisms which mediate the transfer of ions across the root must, however, await the result of detailed biochemical investigations.

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Carbon Dioxide Fixation by Barley Roots[1]

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SUMMARY

The non-volatile, 80 per cent. ethanol-soluble products of fixation have been investigated in excised roots, using $C^{14}O_2$ and radiochromatography.

The main radioactive compounds separated were malic, citric (or *iso*-citric), aspartic, and glutamic acids, asparagine and glutamine.

Less activity was present in serine, tyrosine, α-ketoglutaric acid, and alanine, and in a number of unidentified compounds.

The uptake of $C^{14}O_2$ was inhibited by virtually anaerobic conditions.

From the above observations it is considered likely that C^{14} is transformed through the reactions of the tricarboxylic acid cycle.

C^{14} in the soluble fraction was markedly increased by maintaining the root material in water rather than in a nutrient solution prior to exposure to $C^{14}O_2$. This increase was chiefly in malic acid.

INTRODUCTION

MANY non-green tissues, both plant and animal, are capable of assimilating carbon dioxide (e.g. Ruben and Kamen, 1940; Foster, Carson, Ruben, and Kamen, 1941; van Niel, Ruben, Carson, Kamen, and Foster, 1942; Gollub and Vennesland, 1947; Vennesland, Ceithaml, and Gollub, 1947) and, in a number of heterotrophic micro-organisms, a certain concentration of the gas has been shown to be essential for growth (e.g. Gladstone, Fildes, and Richardson, 1935; Hes, 1938).

Ruben and Kamen (1940), using short-lived radioactive C^{11}, were the first to demonstrate fixation of carbon dioxide by a preparation of barley roots, while the uptake of the bicarbonate ion was studied by Overstreet, Ruben, and Broyer (1940). Owing to the short half-life of the tracer and to the absence at that time of a convenient and rapid micro-method of separation, neither of these groups of workers was able to determine in what compounds the radioactivity appeared and in what sequence.

Following the availability of C^{14}, Laties (1947, 1949) studied fixation of $C^{14}O_2$ by barley roots during malonate inhibition and found the product to be carboxyl-labelled succinic acid. By means of the radiochromatographic technique, which has been used by Lynch and Calvin (1952) in studies of carbon dioxide fixation in various micro-organisms, the present writer has determined the non-volatile, ethanol-soluble products of fixation in barley roots and has

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examined the effects of certain factors on fixation. A preliminary statement of the main results of this work has been published (Poel, 1952).

GENERAL TECHNIQUE

Plant material. Barley seedlings (variety 'Atlas') were grown on wire screens over unaerated tap-water in a greenhouse. At an early stage in the work, seedlings were also grown in the laboratory at 24° C., the purpose being to control more closely the conditions of growth and thereby to produce very uniform material. It was found, however, that those roots grown in the greenhouse were superior for metabolic studies, being more numerous, uniform, and almost devoid of root-hairs.

The roots were excised when 1 week old and placed in aerated Hoagland solution for 24 hours at 24° C. The Hoagland solution (Hoagland and Broyer, 1936) was modified by omitting ferric tartrate, as this tends to precipitate on the roots and it was considered that an iron-deficient medium would not introduce metabolic irregularities during the period of the experiments.

Exposure to $C^{14}O_2$. A quantity of excised roots was removed from the nutrient solution, lightly blotted, and an arbitrarily chosen weight of 0·7 g. taken. The roots in the sample were cut at random into short lengths and placed in a modified Warburg flask. In the preliminary experiments the supporting medium in the flask was fresh Hoagland solution minus iron, but in all the experiments referred to in this paper, 5 ml. of phosphate buffer (pH 5·6) was used in order to maintain a stable reaction during exposure to radioactive carbon dioxide. The side-arm of the flask contained 250 μl. of a solution of $NaHC^{14}O_3$ representing 5·75 μmol. of CO_2 and 51·25 μC. of C^{14}. The vessel was attached to a Warburg manometer and equilibrated for 1 hour at 24° C., with shaking at approximately 130 oscillations per minute. At the end of this period, the stopcock of the manometer was closed and the bicarbonate solution discharged into the supporting buffer. Shaking was continued for 1 hour. By means of the manometer, CO_2 pressure could be checked during the period of exposure and any discrepancies in this respect between the various units of an experiment detected.

Preparation of ethanol extract. Having removed the manometer from the shaker, the flask was opened in a well-ventilated fume cupboard and the buffer decanted off. The roots were rinsed rapidly with distilled water to remove residual salts, likely to interfere with the chromatographic separation, and boiling 80 per cent. ethanol was poured on. Root material, with added ethanol, was then transferred with rinsings of 80 per cent. ethanol to a mortar and thoroughly ground. Quantitative transfer to a stoppered, graduated cylinder followed, the volume of the extract being recorded when cool.

Aliquots of 100 μl. of strongly centrifuged extract were 'plated' on aluminium disks for radioactivity assay, using a thin, end-window Geiger-Muller tube. The activity of the total volume of ethanol extract could thus be calculated.

Radiochromatography. The use of long-lived C^{14} and the radioautography of filter-paper chromatograms render the identification of the compounds in which fixed radiocarbon appears a simple matter. The general technique has been described elsewhere (Calvin, 1949; Benson, Bassham, Calvin, Goodale, Haas and Stepka, 1950). In the present investigation the ethanol extract, after determination of radioactivity, was filtered and concentrated to about 1 to 2 ml. *in vacuo*. Aliquots of the concentrate, varying in different experiments from 100 to 300 μl. according to activity, were placed on sheets (18 in. × 22½ in.) of Whatman No. 1 filter-paper and run two-dimensionally in phenol-water and butanol-propionic acid-water. Radioautographs were prepared on Eastman 'No Screen' X-ray film. As the amount of C^{14} fixed in heterotrophic fixation is small by comparison with photosynthesis, long exposures are frequently required, sometimes of the order of a month or more, depending upon the activity of the aliquot of extract placed on the paper and upon the number of radioactive compounds separated.

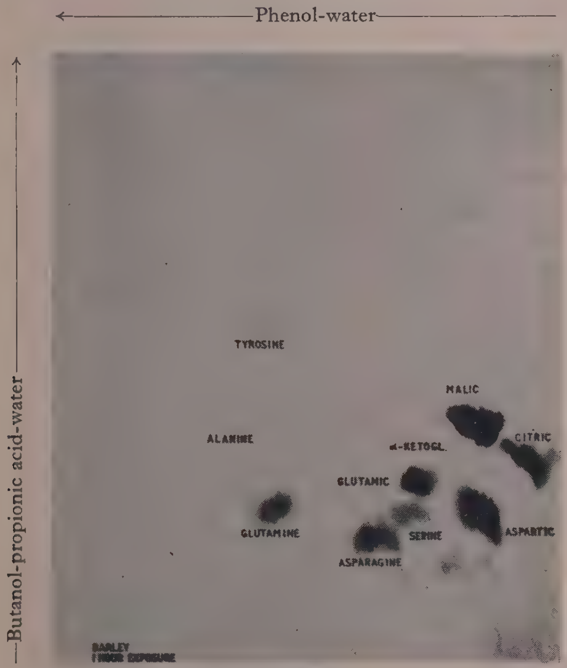

FIG. 1. Radioautograph showing the typical 80 per cent. ethanol-soluble products of $C^{14}O_2$ fixation.

THE PRODUCTS OF FIXATION

The typical non-volatile, 80 per cent. ethanol-soluble compounds found to be radioactive were malic, citric (or *iso*citric), aspartic, and glutamic acids, serine, asparagine, glutamine, and tyrosine (Fig. 1). The identities of all the named radioactive spots have been confirmed by elution and co-chromatography with standard compounds. Unnamed spots have yet to be identified unequivocally. Although alanine produced a large, clear spot on the paper with ninhydrin, it contained little C^{14}. The activity of α-ketoglutaric acid was low and only a trace of radioactive threonine occurred. It is by no means certain, however, that these weakly radioactive compounds are unimportant in the path of fixed carbon, since they may occur only in catalytic concentrations at any given instant. For example, the considerable activity located in glutamic acid and glutamine suggests that a rapid transfer of C^{14} through α-ketoglutaric acid takes place.

The pattern of products in barley roots is very similar to that obtained by Benson and Calvin (1950) for dark aerobic fixation of $C^{14}O_2$ by the leaves of the same plant, except that only a trace of activity appeared in asparagine and none in tyrosine in the experiments with leaves. In both roots and leaves all the compounds are consistent with a sequence of C^{14} transformations through the tricarboxylic acid cycle, previously indicated by the work of Laties (1947, 1949). Aspartic, glutamic, and malic acids were found to be radioactive in all the organisms studied by Lynch and Calvin (1952), but the percentage of the total soluble activity made up by these substances varied widely with different materials. Glutamine and asparagine were rarely encountered and then only in low activity. It would thus appear that although the general pattern of dark fixation is the same in a wide range of organisms and tissues, the relative rates of the intermediate reactions are very diverse.

FIXATION AND MINERAL NUTRITION

When, during the course of the research, phosphate buffer was substituted for modified Hoagland solution as the exposure medium, continued pre-equilibration in the nutrient solution appeared to be illogical, especially as the roots had been grown in tap-water. In later experiments therefore, the roots were placed in aerated tap-water for 24 hours after excision, all other conditions being unchanged.

This treatment resulted in at least five times as much activity in the soluble fraction. A similar effect followed equilibration in distilled water (Table I).

The radioautographs for tap and distilled water are indistinguishable and an example is reproduced in Fig. 2. The characteristic feature is the very obvious predominance of malic acid. This suggests that, as a direct or indirect result of mineral starvation, transformation of C^{14} is impeded. Restriction of amination as a direct consequence of nitrogen deficiency might be a cause of such blockage. Clearly, special significance attaches to the amount and nature of the ethanol-insoluble activity in deciding whether the increased soluble activity following water equilibration represents an overall increase in fixation or, as appears more likely, an accumulation of activity in soluble products. The insoluble residues remaining from these experiments will be investigated. Moreover, it is hoped later to study the entire problem of carbon

TABLE I

The effect of pre-equilibration in Hoagland solution minus iron, tap-water and distilled water on fixation of $C^{14}O_2$ by excised barley roots

Roots: 0·7 g. wet wt. Exposure medium: phosphate buffer, 5 ml. pH 5·6, 250 μl. $NaHC^{14}O_3$ containing 5·75 μmol. CO_2 and 51·25 μC. C^{14}. Time of exposure: 1 hour.

Pre-equilibration medium	Expt. No.	Radioactivity of 80% ethanol extract (counts per min.)	% of activity supplied
Hoagland minus iron	15/1	158,700	1·0
	16/1	129,250	0·8
	16/2	142,220	0·9
	Means:	143,390	0·9
Tap-water	15/2	868,770	5·6
	16/3	783,000	5·0
	16/4	626,240	4·0
	Means:	759,337	4·9
Distilled water	17/3	814,000	5·2
	17/4	982,600	6·3
	Means:	898,300	5·8
Tap-water	17/1	878,080	5·6
	17/2	942,656	6·0
	Means:	910,368	5·8

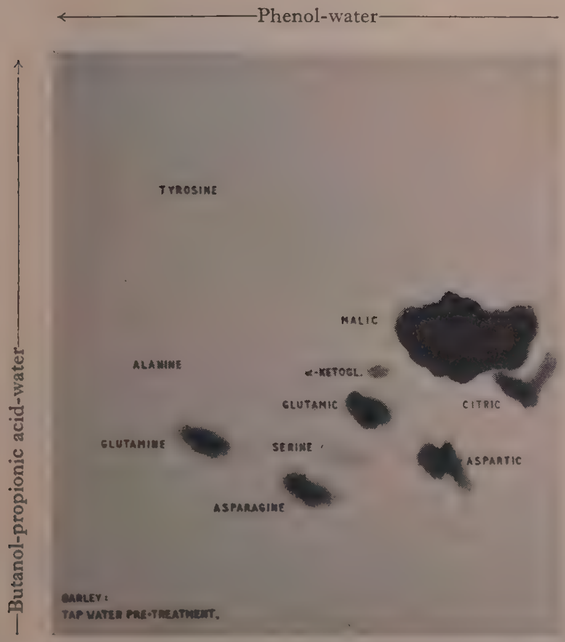

FIG. 2. Radioautograph showing the 80 per cent. ethanol soluble products of $C^{14}O_2$ fixation, following pre-equilibration of the roots in tap-water.

dioxide fixation in relation to mineral nutrition in greater detail, since this may have important bearings on the interrelationships of mineral nutrition and the metabolism of organic acids, carbohydrates, and proteins.

FIXATION AND OXYGEN DEFICIENCY

Further information on how tracer carbon becomes dispersed through the range of compounds revealed in the radioautographs was obtained by studying the effects of virtually anaerobic conditions on fixation.

The same quantities of root material, phosphate buffer, and $NaHC^{14}O_3$ as before were used, the excised roots being pre-equilibrated in tap-water. Cylinder nitrogen (containing approximately 0·5 per cent. oxygen) was passed through the reaction vessel for 30 minutes, following which the stopcocks were closed and the flask immersed in the constant temperature bath (24° C.) with shaking at 130 oscillations per minute. The C^{14}-labelled bicarbonate, introduced into the vessel's side-arm prior to flushing with nitrogen, was then discharged into the buffer and shaking continued for 1 hour. Control experiments were run using air instead of nitrogen.

The results for soluble activity are shown in Table II. No data are yet available for insoluble activity, nor have chromatograms been prepared.

TABLE II

Fixation of $C^{14}O_2$ *by excised barley roots in air and in cylinder nitrogen*

Roots: 0·7 g. wet wt. Exposure medium: phosphate buffer, 5 ml. pH 5·6, 250 μl. $NaHC^{14}O_3$ containing 5·75 μmol. CO_2 and 51·25 μC. C^{14}. Time of exposure: 1 hour.

Atmosphere	Expt. no.	Radioactivity of 80% ethanol extract (counts per min.)	% of activity supplied
Air	20	512,120	3·4
	22	504,240	3·2
	Means:	508,180	3·3
Cylinder nitrogen	21	4,440	0·03
	23	4,480	0·03
	Means:	4,460	0·03

The aerobic nature of the mechanism whereby C^{14} is incorporated appears to be clearly established by these experiments, and this may be interpreted as additional evidence for the operation of the Krebs cycle in barley roots.

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The Relative Toxicity of Manganese and Cobalt to the Tomato Plant

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SUMMARY

1. At equivalent concentrations, cobalt was more effective than manganese in inducing chlorosis and necrosis in tomato plants.

2. Chlorotic plants suffering from either cobalt or manganese toxicity alone, or from the two conditions together, contained as much or more iron than normal plants, and chlorotic leaves responded positively to painting with a solution of ferrous sulphate.

3. When cultures were supplied with 5 p.p.m. Mn, distinct injury of the lower leaves occurred regardless of the cobalt supply, but the addition of cobalt up to 0·01 p.p.m. brought about a gradual decrease in the chlorosis of the top leaves and an increase in dry weight.

4. With cobalt at the 0·1 p.p.m. level, severe chlorosis and necrosis occurred and growth was stunted, but by raising the manganese level to 5·0 p.p.m. a substantial antidoting effect on the toxicity was obtained.

5. Cobalt and manganese had an antidoting effect on each other, whereby at high manganese supplies small quantities of cobalt alleviated manganese toxicity, while toxic concentrations of cobalt were antidoted by high levels of manganese.

INTRODUCTION

IN order to explain the chlorosis developed in soya-bean plants grown at high manganese supplies, Somers and Shive (1942) put forward a hypothesis involving the oxidation by manganese of ferrous iron to ferric iron; the latter reacted with organophosphates and became inactivated. This oxidation was ascribed to a higher oxidation-reduction potential associated with manganese as compared with iron. Since the valency charge of cobalt is associated with a higher potential than that of manganese, cobalt should on this hypothesis be more effective in oxidizing iron than manganese; in an experiment with corn plants, Somers and Shive (1942) showed that iron deficiency was induced more readily by cobalt than by manganese when supplied in equivalent concentrations. It seemed therefore desirable to investigate the relative toxicity of manganese and cobalt and to observe the specific effects of these elements on the growth and development of plants.

EXPERIMENTAL METHODS

The water-culture technique described by Arnon (1938), Stout and Arnon (1939), and recently summarized by Twyman (1951), was used. The nutrient

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[1] The work reported here was carried out at Birmingham University, England.

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solution, as reported by Arnon (1938), containing all the major and trace elements with the exception of manganese and cobalt, was used in all cultures. Iron was supplied as ferric citrate at the rate of 0·5 p.p.m. in the medium. In the first experiment manganese and cobalt were supplied in equivalent concentrations; those of manganese were 0·0, 0·25, and 10·0 p.p.m., while the corresponding equivalent concentrations of cobalt were 0·0, 0·27, and 10·7 p.p.m. The culture solutions were changed at fortnightly intervals. Fifty-four cultures were set up in this experiment, including nine treatments with sixfold replication of each. The tomato seeds were set to germinate on 24 September 1948, and a fortnight later 54 seedlings were transplanted to culture vessels containing only the basic culture solution (i.e. with no Mn or Co). The treatments were started on 22 October, and the plants were cropped on 3 December, having received 6 weeks' treatment. After cropping each plant was divided into root and shoot systems, and the fresh and dry weight of each system determined. The shoot systems were subsequently analysed for iron and manganese; the method used for the estimation of iron was the thioglycollic acid method (B.D.H. reagents for delicate analysis, 1939), while the potassium periodate method (Snell and Snell, 1945; Piper, 1947) was used for estimating manganese.

In the second experiment four different concentrations of manganese and cobalt were used. Those of manganese were 0·0, 0·05, 0·25, and 5·0 p.p.m., while cobalt was supplied at the following levels: 0·0, 0·001, 0·01, and 0·1 p.p.m. Sixty-four cultures were used, there being 16 treatments and 4 replicates of each treatment. Iron was again supplied at the rate of 0·5 p.p.m. The seeds were planted out into the culture vessels on 6 January 1949. The differential treatments were started on 27 January when the plants were fairly well developed (i.e. fourth-leaf stage). The plants were harvested on 15 March, having received 46 days' treatment. The fresh- and dry-weight yields were determined and the tissues analysed for iron and manganese as described above.

RESULTS OF THE FIRST EXPERIMENT

Observations

(*a*) *Manganese toxicity*. The cotyledons became yellow with purple tints; the newly developed leaves gradually became yellow and later developed brown necrosis; the older leaves developed minute purpled spots along the veins and eventually became yellow and withered up; the lower leaves died first; the roots, however, remained normal, but were reduced in size. This condition was observed at the 10·0 p.p.m. manganese supply. At the 5 p.p.m. Mn level the symptoms were less severe.

(*b*) *Cobalt toxicity*. Two levels of toxicity were recognized: (i) at 0·1 and 0·27 p.p.m. Co, and (ii) at 10·7 p.p.m. Co. At the lower levels of cobalt supply the symptoms were as follows: the plants were stunted, the top leaves, followed by the lower ones, became yellow; the newly developed leaves were reduced in size and entirely yellow in colour but later developed brown necrosis; the affected leaves bent downwards and finally became scorched; the development of purplish tints on the stems, veins, and lower surface of affected leaves together with brown and reduced root systems were characteristic.

At the highest cobalt supply the whole plant was very rapidly affected, as shown by a severe chlorosis and necrosis, followed later by an upward rolling and scorching of the leaves; the growth was severely checked both of the shoot and root systems; the latter were particularly poorly developed and acquired a brown coloration; death of these plants was inevitable.

(*c*) *Manganese deficiency*. The development of a chlorotic mottling on the upper mature leaves was the first sign of manganese deficiency. The chlorosis extended to the lower leaves and eventually appeared with the newly formed leaves. Later minute necrotic spots appeared in the intervenal areas.

(*d*) *Iron deficiency*. Early symptoms were first apparent in the terminal leaves where an intervenal chlorosis developed at the base of the leaflets and soon extended to the tips. Newly formed leaves were more chlorotic, and finally they were wholly yellow with purplish veins. Growth was stunted, and leaf size and leaf production were much reduced.

In the first experiment the cultures not supplied with cobalt showed the best growth as compared with those grown at the two cobalt levels. All the cultures not receiving manganese showed manganese deficiency symptoms, while those receiving 0·25 p.p.m. Mn were quite normal and gave the highest yields. The cultures given 10·0 p.p.m. Mn exhibited manganese toxicity.

At the 0·27 p.p.m. Co level the first symptoms of toxicity appeared 3 days after starting the treatments irrespective of the manganese supply. The plants supplied with 10·0 p.p.m. Mn at this level of cobalt had much better growth than those receiving 0·25 p.p.m. Mn, although manganese and cobalt toxicity symptoms were apparent. There was evidence therefore of manganese reducing the toxic action of cobalt.

Plants supplied with 10·7 p.p.m. Co with or without manganese exhibited symptoms of severe cobalt injury. In the case of cultures supplied with manganese, the withering of the leaves was somewhat delayed, but at harvest all the plants were uniform in respect to the visual symptoms of cobalt injury.

TABLE I

A comparison of the dry weight yield (mg.) based on the evaluation of Student's 't'

Mn conc. in medium	Co concentration in medium (p.p.m.)			Significance at 5% level
(p.p.m.)	0·0	0·27	10·7	
0·00	A 812	B 125	C 103	A > B > C
0·25	D 2005	E 246	F 102	D > E > F
10·00	G 611	H 323	I 103	G > H > I
Significance at 5% level	D > A = G	H = E > B	C = F = I	

Thus from a consideration of the general appearance and growth of the plants it was clear that cobalt was much more toxic than manganese at equivalent concentrations, and that high manganese nullified to some extent the toxic action of cobalt.

TABLE II

A classification of the treatments based on the descending order of the average dry and fresh weights, height, and number of leaves per plant

Group	Treatment letter	Mn conc. in the medium (p.p.m.)	Co conc. in the medium (p.p.m.)	Average per plant: F.W. (g.)	D.W. (mg.)	Height (cm.)	No. of leaves	Condition at harvest
1	D	0·25	0·0	29·11	2005	34·6	6·3	Healthy
	A	0·00	0·0	13·59	812	26·2	6·0	Mn def.
	G	10·00	0·0	9·10	611	18·9	6·0	Mn tox.
2	H	10·00	0·27	3·52	323	14·5	4·5	Medium Co toxicity
	E	0·25	0·27	2·75	246	12·5	4·8	
	B	0·00	0·27	1·42	125	9·7	4·0	
3	C	0·00	10·7	0·75	103	9·2	3·5	Severe Co toxicity
	F	0·25	10·7	0·87	102	8·2	3·6	
	I	10·00	10·7	0·67	103	7·9	3·3	

Fresh- and dry-weight yields

Fresh- and dry-weight data are summarized in Table II, while Table I indicates the results of statistical treatment of the dry-weight yields. It is evident from a consideration of these two tables that cobalt was more toxic than manganese at equivalent concentrations. If the fresh- and dry-weight yields and the height and number of leaves are arranged in descending order, it is found that the results fall naturally into three groups as shown in Table II. Group I includes the treatments where the plants received no cobalt. The progressive decrease in dry weight, height, and number of leaves per plant was due first to lack of manganese (treatment A) and then to manganese toxicity (treatment G). In the second group the progressive decrease in growth and development per plant was due to the presence or absence of manganese. The presence of high manganese appeared to alleviate the effect of cobalt (treatment H compared with treatments E and B). Thus it is seen that the two toxic elements do not operate together with respect to their effect on the tomato plant. In the third group all cultures received 10·7 p.p.m. Co and severe cobalt toxicity resulted, the presence or absence of manganese having no effect.

The total iron and manganese content of the tissues

The analytical data for iron and manganese are shown in Table III. From Table III it can be seen that, at each level of manganese supply, increasing the cobalt concentration in the medium increased the iron concentration in the tissues. Thus the most chlorotic plants (treatments C, F, and I) contained more iron than the normal plants (treatment D). This was also the case when

TABLE III

Average iron and manganese concentrations (p.p.m.) in the dry tissues at harvest

Mn conc. in medium p.p.m.	Co concentration in the medium (p.p.m.)					
	0·0		0·27		10·7	
	Mn	Fe	Mn	Fe	Mn	Fe
0·0	5	120	5	200	9	245
0·25	244	120	120	169	34	273
10·00	2785	167	1753	239	764	334

the manganese supply was increased at the two cobalt levels (treatments H and I). The chlorosis caused by manganese or cobalt was cured by painting the chlorotic areas with 0·25 per cent. ferrous sulphate solution in a wetting reagent. It seemed therefore that a considerable proportion of the iron in the leaves was 'inactivated' and that high concentrations of manganese (10·0 p.p.m.) and much lower ones of cobalt (0·27 p.p.m.) were responsible for the inactivation.

Except for the cultures deprived of manganese, the manganese concentration in the tissues was depressed as the cobalt concentration in the medium increased. At each cobalt level, increasing the supply of manganese increased the concentration of manganese in the tissues (Table III). Increasing the manganese supply at the 0·27 p.p.m. Co level (treatments B, E, and H) caused not only an increase in the manganese concentration in the tissues but also brought about an increase in yield and a decrease in the cobalt injury. Thus manganese had an antidoting effect on cobalt.

Since the levels of cobalt used in this first experiment were so toxic, and since some evidence was obtained to substantiate a Mn/Co interrelationship, it was decided to carry out a second experiment at lower cobalt concentrations.

RESULTS OF THE SECOND EXPERIMENT

The effect of maintaining constant manganese supply and varying the cobalt supply. In the absence of manganese, all plants of treatments A, B, and C receiving 0·0, 0·001, and 0·01 p.p.m. cobalt respectively developed severe symptoms of manganese deficiency, but at the high cobalt supply (0·1 p.p.m. treatment D) these symptoms were obscured by those of cobalt toxicity. The trend of dry-weight yields with increasing cobalt supply was that no significant effect was obtained until 0·1 p.p.m. Co was supplied (see Table V). This concentration was very toxic.

In the case of plants grown at the 0·05 p.p.m. and 0·25 p.p.m. manganese, the effect of increasing the cobalt supply was to reduce the height of the stems, the length of leaves, and the dry-matter production (Tables IV and V, treatments E, F, G, and H and treatments I, J, K, and L). As regards visual ymptoms of injury, however, only those cultures supplied with 0·10 p.p.m. o showed the chlorotic and necrotic symptoms of cobalt toxicity.

At the 5·0 p.p.m. Mn level (treatments M, N, O, and P) all the plants respective of the cobalt supply showed the purple necrosis along the veins of

TABLE IV

l comparison of the dry-weight yield (g.) based on the evaluation of Student's 't'

Mn conc. in the medium p.p.m.	Co concentration in the medium (p.p.m.)				Significance at 5% level
	0·0	0·001	0·01	0·1	
0·05	A 1·551	B 1·198	C 1·354	D 0·207	A = B < C > D, A = C
0·05	E 3·964	F 2·187	G 1·918	H 0·236	E > F = G > H
0·25	I 3·770	J 3·398	K 3·090	L 0·280	I = J = K > L
5·00	M 1·803	N 2·015	O 2·544	P 1·288	M = N < O > P, M = P, N > P
Significance at 5% level	A < E = I > M, A = M	B < F < J > N, B < N	C < G < K = O, C < O	D = H = L < P	

TABLE V

lverage total fresh and dry weights, height of stems, length of seventh leaf on each plant and number of leaves per plant at harvest

Treatment	Mn conc. in medium (p.p.m.)	Co conc. in medium (p.p.m.)	Average D.W. (g.)	Average height (cm.)	Average leaf length (cm.)	Average No. of leaves
A	0·00	0·000	1·551	22·2	15·5	11·0
B	0·00	0·001	1·198	21·2	14·5	10·0
C	0·00	0·010	1·354	22·3	18·0	11·0
D	0·00	0·100	0·207	7·5	5·1	8·8
E	0·05	0·000	3·964	29·7	21·5	12·5
F	0·05	0·001	2·187	22·7	19·5	10·8
G	0·05	0·010	1·918	20·5	18·5	11·0
H	0·05	0·100	0·236	9·3	6·0	8·8
I	0·25	0·000	3·770	32·1	24·2	13·0
J	0·25	0·001	3·398	28·5	23·0	11·7
K	0·25	0·010	3·090	26·8	22·1	11·5
L	0·25	0·100	0·280	9·5	7·0	8·8
M	5·00	0·000	1·803	18·1	17·0	11·5
N	5·00	0·001	2·015	21·2	17·3	11·0
O	5·00	0·010	2·544	25·5	19·1	11·0
P	5·00	0·100	1·288	19·8	15·0	12·3

he lower leaves characteristic of manganese toxicity. Increasing the cobalt upply up to 0·10 p.p.m. brought about an increase in yield and a decrease in he severity of the chlorosis due to manganese toxicity. Plants receiving ·1 p.p.m. Co were very small and showed severe cobalt toxicity. Thus cobalt n very low concentration (0·001 and 0·01 p.p.m.) appeared to alleviate the oxic action of manganese.

The effect of maintaining the supply of cobalt constant and varying the manganese supply. This effect is seen by considering the results in Table IV in a vertical direction. When cobalt was given in low concentration (0·0 to 0· p.p.m.) the best supply of manganese was 0·25 p.p.m. Perhaps the most interesting effect was that obtained at the high cobalt level (0·1 p.p.m.); wi increasing manganese from 0·0 to 5·0 p.p.m. there was an increase in d weight, and at the highest manganese supply the symptoms of cobalt toxici were reduced. Similar results were recorded in the first experiment.

Leaf painting. The chlorosis of leaves caused by toxic quantities of manganese or cobalt, or a mixture of both, could be cured by painting the leav with 0·25 per cent. ferrous sulphate in a wetting agent. The yellow leaves became green in four days after treatment.

The iron and manganese content of the tissues. Table VI records the averag iron and manganese concentrations in the tissues. It is evident from Table V that the iron concentration in the tissues was not related to the incidenc of manganese deficiency, manganese toxicity, or cobalt toxicity. Chlorot plants suffering from manganese or cobalt toxicities, or both together, contained as much or more iron than normal green plants.

Increasing the supply of manganese increased the concentration of manganese in the tissues. If the results in Table VI are considered horizontally it seen that increasing the cobalt supply at the 0·05 and 0·25 p.p.m. Mn leve increased the manganese concentration in the tissues. This result could b explained by assuming that the effect of cobalt was to reduce the growth an development of the plant, while the total amount of manganese absorbe remained almost constant. This is shown to be the case in Table VII, whe the absolute amounts of manganese in the tops has been calculated, and approximately the same in each case. Thus in spite of the decrease in dry weight yields of the plants (Table VII, treatments E, F, G and I, J, K increasing the cobalt at these two manganese levels did not depress the absorption of manganese. In the case of plants given 5·0 p.p.m. Mn (Table VI the progressive decrease in manganese injury by increasing the cobalt suppl from 0·0 to 0·01 p.p.m. was accompanied by a decrease in the manganes concentration in the tissues and an increase in the dry-weight yields. This increase in dry-weight yield, however, was not accompanied by an increase i

TABLE VI

Average iron and manganese concentrations (p.p.m.) in the dry tissues

Mn conc. in the medium (p.p.m.)	Co concentration in the medium (p.p.m.)							
	0·0		0·001		0·010		0·100	
	Mn	Fe	Mn	Fe	Mn	Fe	Mn	Fe
0·00	1*	113*	1*	137*	1*	108*	0*	122†
0·05	36	96	56	109	61	104	145†	113†
0·25	101	120	124	161	162	101	445†	132†
5·00	976§	128§	902§	132§	784§	125§	1264†§	118†§

* Mn-deficient. † Co-toxicity. § Mn-toxicity.

TABLE VII

Showing the variation in manganese concentration (p.p.m.) and the total manganese (mg.) in the green tissues of tomato plants at three levels of manganese supply, as the cobalt concentration in the medium increased

Treatment	Mn conc. in medium (p.p.m.)	Co conc. in medium (p.p.m.)	Av. dry wt./plant (g.)	Mn conc. in tops (p.p.m.)	Total Mn in tops (mg.)
E	0·05	0·000	3·964	36	0·122
F		0·001	2·187	56	0·102
G		0·010	1·981	61	0·098
I	0·25	0·000	3·770	101	0·322
J		0·001	3·398	124	0·354
K		0·010	3·090	162	0·424
M	5·00	0·000	1·803	976	1·415
N		0·001	2·015	902	1·488
O		0·010	2·544	784	1·644

the total manganese absorbed corresponding to the increased yield. There was nothing in the analytical data to show whether the progressive decrease in manganese injury (at the 5 p.p.m. Mn level) was due solely to the decreased manganese concentration within the tissues, or to cobalt acting as an antidoting agent on manganese within the tissues, and thereby stimulating better growth.

At the highest cobalt supply (0·1 p.p.m.) the manganese concentration in the tissues was always higher than at the lower cobalt levels (Table VI). This was due to the decrease in dry weight caused by the toxic action of the cobalt, and to the apparent uncontrolled absorption of manganese under these conditions.

DISCUSSION

There was evidence in these experiments indicating that manganese reduces the toxic effect of cobalt. For example, the retardation in the growth and development of cultures supplied with 0·25 p.p.m. Mn was less than in those supplied with 0·05 p.p.m. Mn under the action of additional cobalt (Table VII); further, the higher the manganese level in the medium, the better the growth and development of cultures given 0·1 p.p.m. Co (Table IV). The reverse reaction also takes place, i.e. cobalt reduced the toxicity of manganese, but in this case only very small amounts of cobalt were needed to depress the toxicity of manganese (Table VII, 5·0 p.p.m. Mn and 0·0 to 0·01 p.p.m. Co). Thus with regard to manganese toxicity, small quantities of cobalt were needed to depress this toxicity, while relatively greater amounts of manganese were required to antidote only partially cobalt toxicity. Antidoting effects of this nature have been reported by Liebig, Vanselow, and Chapman (1942), who showed that copper toxicity in *Citrus* could be alleviated by the addition of aluminium to the culture medium. Lewis and Powers (1941) found a similar interrelationship between chlorides and iodides in corn plants.

With regard to the effect of cobalt and manganese on iron metabolism, it would appear that cobalt inactivates iron much more effectively that manganese at equivalent concentrations. The explanation of this effect, however, must be more complex than the oxidation-reduction hypothesis put forward by Somers and Shive (1942). This hypothesis has been criticized by Arnon (1943) and Hewitt (1948, 1949). The chlorosis reminiscent of iron deficiency was only a phase in the development of the symptoms of cobalt toxicity which, like those of manganese toxicity, were quite distinct from iron deficiency. Thus both cobalt and manganese, while affecting iron metabolism, had other independent effects as well.

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The Krebs Cycle Enzyme System of Pea Seedlings

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SUMMARY

A particulate fraction, showing catalytic activity towards intermediates of the Krebs cycle, has been isolated from pea seedlings by the method of differential centrifugation. The particulate material, possibly consisting of mitochondria, appears homogeneous under the microscope and is stained by Janus Green B.

The enzyme system necessary for the integrated and single step reactions of the Krebs tricarboxylic acid cycle is described, and the requirements for cofactors, discussed.

INTRODUCTION

A CONSIDERABLE body of evidence is available which supports the view that a Krebs cycle is functional in animal tissues (Krebs, 1943). The evidence for the operation of the cycle in plants is largely indirect, due in part to the presence of high concentrations of one or more of the substrate acids in the vacuole, and in part to the low metabolic activity of mature plant cells.

The development of centrifugation methods for the isolation of the cyclophorase system (Green, Loomis, and Auerbach, 1948) and mitochondria (Hogeboom, Schneider, and Pallade, 1948) has made possible a direct attack on the problem of the Krebs cycle enzyme system of plants. Several workers (Bhagvat and Hill, 1951; Stafford, 1951; Meeuse, 1952) have isolated particuate material from plants, but cytochrome *c* was found to be necessary for succinoxidase activity, and the malic oxidase system required co-enzyme I in addition to cytochrome. Recently, Millerd, Bonner, Axelrod, and Bandurski, (1951) isolated particulate material, which consisted of mitochondria, from seedlings of the mung bean *Phaseolus aureus*. Adenosine-triphosphate and Mg ions were the only requirements for full activity in the complete oxidation of pyruvic acid.

The present work, which was begun in 1950, deals with the properties of particulate material isolated from pea seedlings.

METHODS

Isolation of the enzyme system. The method is based on the procedure of Kennedy and Lehninger (1949) for the isolation of rat liver mitochondria. Peas were soaked overnight and allowed to germinate in the dark for 3 days. The seedlings were thoroughly washed, the cotyledons removed, and a homogenate prepared by grinding the seedlings (20 g.) in a pestle and mortar with sand

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(10 g.) and 25 ml. of a solution (I) containing glucose (0·4 M.) and nicotinamide (0·02 M.). The mortar was chilled to −5° C. before use, and the temperature of the homogenate kept below 3° C. during grinding. Grinding was continued for 3 minutes, the pH being checked with bromothymol blue and adjusted to 7–7·5 by the addition of ice-cold Na_2HPO_4 (0·1 M.). The brei was strained through muslin and centrifuged for 15 minutes at 1,700 × *g*. The supernatant was collected and 0·1 volume of 10 per cent. KCl added. After standing in an ice bath for 5 minutes, the suspension was centrifuged for 30 minutes at 7,500 × g. The supernatant was discarded, the precipitate washed with a solution (II) containing glucose (0·4 M.), nicotinamide (0·02 M.), KCl (1 per cent.), and phosphate buffer (0·05 M.) at pH 7, and re-sedimented by centrifuging for 20 minutes at 7,500 × g. The washing and sedimentation were repeated and the precipitate taken up in 5 ml. of solution II.

All centrifugations were carried out in a refrigerator at 0° C.

Chemicals. Cytochrome *c* was isolated from horse heart by the method of Keilin and Hartree (1945). Diphosphopyridine-nucleotide (DPN) was isolated from yeast by phenol extraction, followed by lead and silver precipitation as outlined by Le Page (Umbreit, Burris, and Stauffer, 1949). The following substances were prepared according to the methods devised by the authors indicated: Trans-aconitic acid (Bruce, 1937), *cis*-aconitic acid (Krebs and Eggleston, 1944), *dl-iso*citric acid (Pucher and Vickery, 1946), oxalacetic acid (Krampitz and Werkman, 1941), sodium pyruvate (Umbreit *et al.*, 1949). All other materials were obtained commercially.

Analytical. Manometric measurements of oxygen uptake were made at 30° C., using the Warburg apparatus with air in the gas space. The flasks were equilibrated for 5 minutes prior to closing of the taps.

Citric acid was determined by following the procedure of Hargreaves, Abrahams, and Vickery (1951), for the preparation of pentabromacetone, which was then determined colorimetrically with sodium sulphide.

Keto acids were determined by the methods of Friedemann and Haugen (1943).

Organic acids were separated and identified by the method of Lugg and Overell (1948), and by the modifications of this method introduced by Stark, Goodban, and Owens (1951). The papers were sprayed with slightly alkaline solutions of bromocresol purple or bromophenol blue, as this proved to be the most sensitive method for detection of the acid spots. However, for photographic records a solution containing starch (0·5 per cent.), KI (15 per cent.), and KIO_3 (4 per cent.), was used for spraying the papers. The acids were identified by their R_f values and whenever possible by the following special spraying reagents:

(1) The Furth-Hermann reagent (acetic anhydride 8 volumes, pyridine 1 volume). This reagent reacted with tricarboxylic acids to give yellow to red spots showing fluorescence in ultra-violet light.

(2) 0·1 per cent. semicarbazide hydrochloride followed by 0·15 per cent. sodium acetate (Magasanik and Umbarger, 1950). The papers were

dried at 110° C. and examined under ultra-violet light. Keto acids were identified as dark spots against a fluorescing background.

(3) 0·5 per cent. osmic acid. After standing overnight unsaturated acids appeared as black spots.

RESULTS

The active material which was thrown down as a pale brown precipitate as readily stained with Janus Green B (1 in 30,000). Under the phase-ontrast microscope the material appeared to consist of spherical particles pproximately 1μ in diameter.

The integrated system. The activity of the enzyme system with various acids f the Krebs cycle was determined by the manometric estimation of oxygen ptake. The results of two experiments are presented in Table I.

TABLE I

tilization of organic acids by a particulate fraction isolated from pea seedlings. ach flask contained: 1 ml. enzyme suspension, glucose (0·3 M.), phosphate uffer pH 7 (0·05 M.), nicotinamide (0·01 M.), ATP (0·003 M.), magnesium hloride (0·005 M.), and substrate acid as indicated in a total volume of 2·5 ml. Alkali in the centre well (0·1 ml.), air in the gas space, and bath temperature 30° C.

	Substrate	Oxygen uptake μM/2 hrs.
1	Pyruvate (0·25 M.)	4·6
	l-Malate (0·025 M.)	6·9
	l-Malate (0·001 M.)	3·8
	Pyruvate (0·025 M.)+*l*-Malate (0·001 M.)	7·6
	None	0·9
2	*l*-Malate (0·025 M.)+Acetate (0·025 M.)	8·3
	dl-*Iso*citrate (0·05 M.)	10·1
	a-Ketoglutarate (0·025 M.)	9·2
	Succinate (0·025 M.)	8·7
	None	1·2

The results indicate that although pyruvic acid was oxidized by the enzyme system, the addition of a small amount of malic acid gave a greatly increased rate of oxygen uptake. The affect of malic acid on the rate of disappearance of pyruvic acid was studied over a period of 2 hours. The results of the experiment are given in Table II.

TABLE II

Utilization of pyruvic acid by the enzyme system. Each tube contained 1·5 ml. suspension, and the reaction mixture given in Table I in a total volume of 3 ml.

Substrate	Loss of pyruvic acid (μg.)
Pyruvate (0·025 M.)	−203
Pyruvate (0·025 M.)+*l*-Malate (0·001 M.)	−320
l-Malate	0

Organic acids were oxidized by the enzyme system in the absence of external cofactors. However, the oxygen uptake was greatly increased by the addition of ATP and Mg ions (Fig. 1). Cytochrome *c* did not increase the oxygen uptake. The affect of DPN on oxygen uptake was found to vary with the substrate (Fig. 2). The affect of DPN on the oxidation of *l*-malate+acetate by the enzyme system was studied by measuring the rate of oxygen uptake and the rate of keto-acid formation. The results of this experiment are illustrated in Fig. 3.

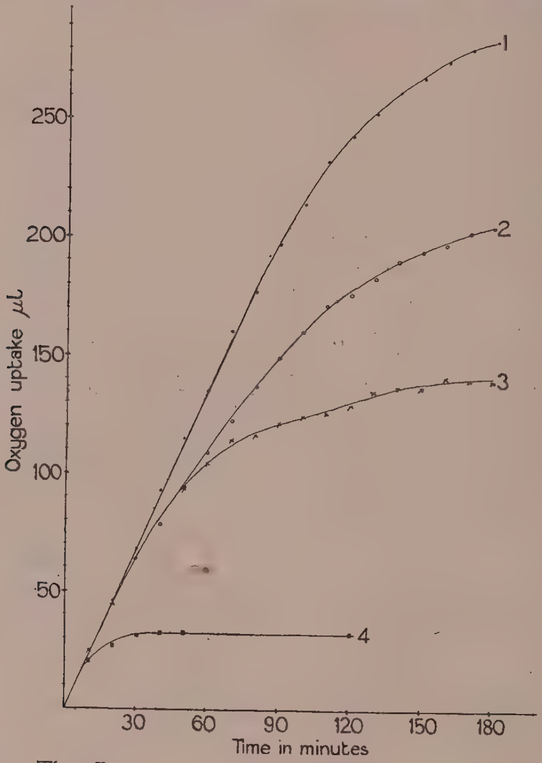

FIG. 1. The effect of ATP and Mg ions on the oxidation of citric acid by the particulate fraction. The complete system consisted of 1 ml. enzyme suspension, glucose (0·3 M.), phosphate buffer pH 7 (0·05 M.), nicotinamide (0·01 M.), sodium citrate (0·025 M.), ATP (0·003 M.), and magnesium chloride (0·005 M.), in a total volume of 2·5 ml. Curve (1) complete system. Curve (2) without Mg. Curve (3) without ATP. Curve (4) without citrate.

Since the increased formation of keto acids was greater than could be accounted for by the increased oxygen uptake, the increased oxidation of *l*-malate must have been at the expense of other oxidations catalysed by the system. Consequently the reaction

$$l\text{-malate} \underset{+2\mathrm{H}}{\overset{-2\mathrm{H}}{\rightleftharpoons}} \text{oxalacetate} \longleftrightarrow \text{pyruvate} + \mathrm{CO_2}$$

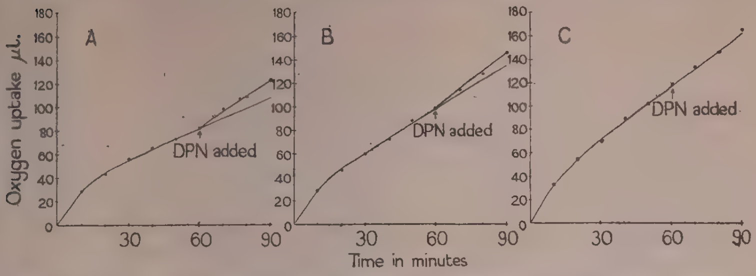

FIG. 2. The effect of DPN on the oxidation of (A) Malate, (B) Malate+acetate, (C) Malate+pyruvate.

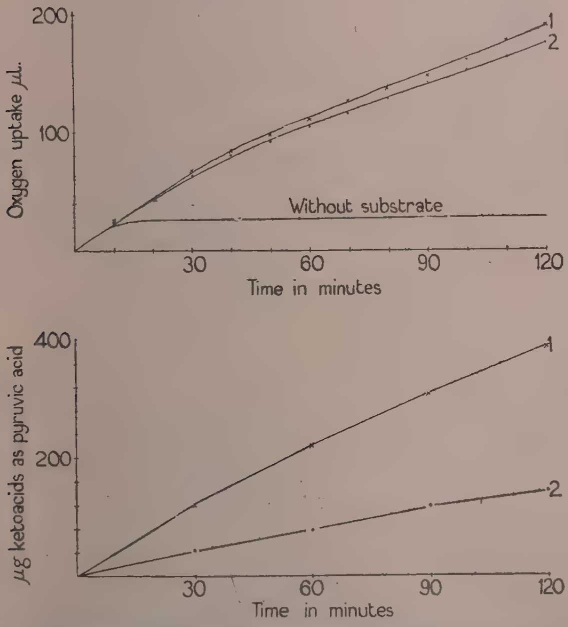

FIG. 3. The effect of DPN on the oxidation of malate+acetate. Curves (1) with DPN. Curves (2) without DPN.

was studied in the presence of other substrates, isocitrate, and succinate (Fig. 4).

*Iso*citrate and succinate decreased the accumulation of keto acids, presumably by supplying their dehydrogenases with electrons and so competing with malic dehydrogenase for the terminal oxidase.

The single-step reactions of the Krebs cycle. The individual steps in the oxidation of pyruvic acid were studied by the methods of Green *et al.* (1948).

$$\text{1.}\quad \text{Citrate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \textit{Cis}\text{-Aconitate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \textit{Iso}\text{citrate.}$$

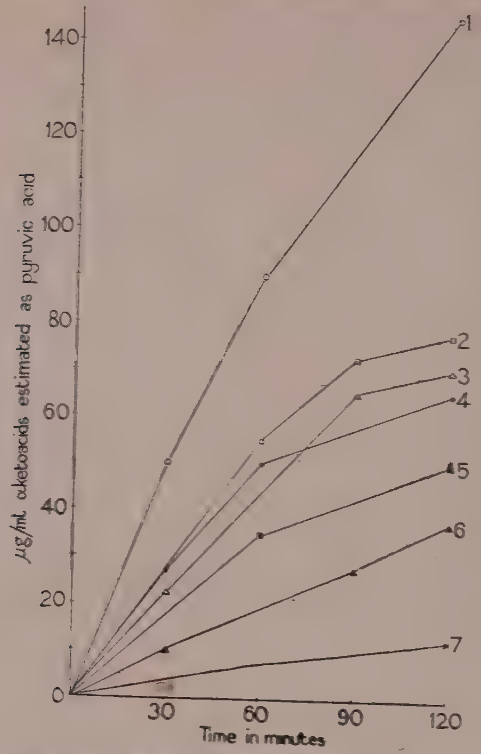

Fig. 4. The effect of various substances on the formation of ketoacids by the system enzyme+malate+acetate. (1) ○——○ with DPN. (2) □——□ with DPN+*iso*citrate. (3) △——△ with DPN+succinate. (4) ●——● system without additions. (5) ■——■ with *iso*citrate. (6) ▲——▲ with succinate. (7) ×——× enzyme+*iso*citrate.

This reaction was studied under anaerobic conditions. The formation of citrate from *cis*-aconitate was demonstrated by means of filter-paper chromatography (Fig. 5). The rate of formation of citrate from *iso*citrate and from *cis*-aconitate was determined by chemical analysis (Table III).

TABLE III

Formation of citrate from isocitrate *and from* cis-*aconitate. Each tube contained 2·5 ml. enzyme suspension, glucose* (0·3 M.), *phosphate buffer pH 7* (0·05 M.), *and 2·69 mg.* cis-*aconitic or 5·82 mg.* dl-iso*citric acid in a total volume of 8 ml.*

Citric acid formed in μg./ml. with substrate indicated	Time in minutes			
	0	60	120	150
Cis-Aconitic acid 2μM./ml.	0	170	260	260
*dl-Iso*citric acid 4μM./ml.	0	140	240	245

2. *Iso*citrate $\underset{2H}{\overset{-2H}{\rightleftarrows}}$ Ketoglutarate+CO_2.

At pH 9 *iso*citrate was oxidized by the enzyme suspension with an accumulaion of α-ketoglutarate. DPN produced a further accumulation of the keto acid. The results of this experiment are presented in Table IV.

TABLE IV

Formation of α-ketoglutarate from isocitrate. *Each tube contained 1 ml. enzyme uspension, phosphate buffer or borax buffer (0·05 M.), ATP (0·003 M.),* $MgCl_2$ *(0·001 M.), and DPN (2 mg.) where indicated, in a total volume of 3 ml.*

Conditions	Ketoglutarate formed in 2 hrs. (μg./ml.)
0·1 M. *dl-Iso*citrate pH 7	10
0·1 M. *dl-Iso*citrate+DPN. pH 7	15
0·1 M. *dl-Iso*citrate pH 9	90
0·1 M. *dl-Iso*citrate+DPN. pH 9	185

$$3.\ \alpha\text{-Ketoglutarate} \xleftrightarrow[2\text{H}]{-2\text{H}} \text{Succinate}+CO_2.$$

This reaction was studied by adding malonic acid to the reaction mixture, thus preventing the oxidation of succinic acid. It was observed that succinate accumulated in the absence of malonate, when the substrate concentration was high (0·1 M.). The substrates and products of the reaction were isolated by filter-paper chromatography (Fig. 5).

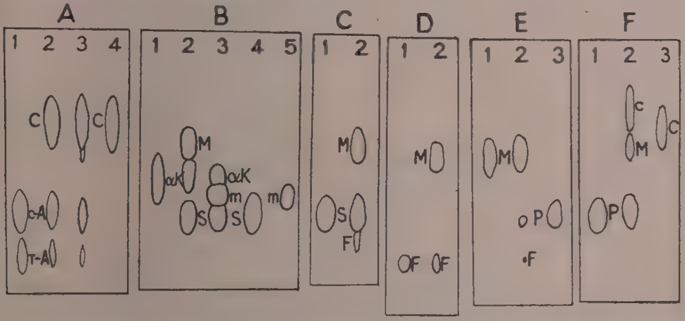

FIG. 5. Chromatographs demonstrating reactions catalysed by the particulate material. (A) Aconitase activity under anaerobic conditions; (1) boiled enzyme+*cis*-aconitate; (2) enzyme +*cis*-aconitate after 1 hour; (3) enzyme+*cis*-aconitate after 2 hours; (4) citrate marker. (B) α-Ketoglutaric oxidase activity; (1) boiled enzyme+α-ketoglutarate; (2) enzyme+α-ketoglutarate; (3) enzyme+α-ketoglutarate+malonate; (4) succinate marker; (5) malonate marker. (C) Succinoxidase activity; (1) boiled enzyme+succinate; (2) enzyme+succinate. (D) Fumarase activity under anaerobic conditions; (1) boiled enzyme+fumarate; (2) enzyme +fumarate. (E) Malic oxidase activity; (1) boiled enzyme+malate; (2) enzyme+malate; (3) Pyruvate marker. (F) Citrogenase activity; (1) boiled enzyme+oxalacetate+pyruvate; (2) enzyme+oxalacetate+pyruvate; (3) citrate marker.

$$4.\ \text{Succinate} \xleftrightarrow[2\text{H}]{-2\text{H}} \text{Fumarate}.$$

This reaction was studied by a variety of treatments. Thus when the enzyme system was exposed to laboratory temperatures in the absence of substrate, succinoxidase activity remained although the other oxidase systems of the

Krebs cycle were inactivated. Succinic-dehydrogenase activity was also retained in fractions isolated by centrifugation at laboratory temperature. These treatments did not destroy fumarase activity, so that malate appeared as a product in the oxidation of succinate (Fig. 5).

$$5.\ \text{Fumarate} \underset{-H_2O}{\overset{H_2O}{\rightleftarrows}} \text{Malate.}$$

This reaction was studied under anaerobic conditions. With fumaric acid as the substrate, the formation of malate was demonstrated by filter-paper chromatography (Fig. 5).

$$6.\ \text{Malate} \underset{-2H}{\overset{2H}{\rightleftarrows}} \text{Oxalacetate.}$$

Oxalacetate was rapidly decarboxylated by the enzyme suspension, consequently it was necessary to trap the oxalacetate with cyanide (Green and Williamson, 1937). Methylene blue was added as an electron acceptor, and the oxidation of *l*-malate followed by the Thunberg method (Table V). The formation of pyruvic acid from malic acid was demonstrated by paper chromatography (Fig. 5).

TABLE V

Malic dehydrogenase activity. Each tube contained 1 ml. enzyme suspension, glucose (0·3 M.), phosphate buffer pH 7 (0·05 M.), nicotinamide (0·01 M.), ATP (0·003 M.), $MgCl_2$ *(0·005 M.), methylene blue (1 in 10,000), and substrate as indicated in a total volume of 5 ml.*

Substrate	Time required for ⅔ reduction of methylene blue	Time required for complete reduction
l-Malate (0·05 M.)	5 minutes	30 minutes
l-Malate (0·05 M.)+KCN (0·002 M.)	6 ”	10 ”
dl-Isocitrate (0·1 M.)	7 ”	10 ”
None	105 ”	—

$$7.\ \text{Oxalacetate}+\text{‘Active’ Acetate} \rightleftarrows \text{Citrate.}$$

It was possible to demonstrate citrate synthesis from oxalacetate in the presence of $MgCl_2$. Pyruvate and acetate increased the amount of citrate formed, but in all cases the yield was small (Table VI).

TABLE VI

Formation of citrate from oxalacetate. Each tube contained glucose (0·3 M.), phosphate buffer pH 7·2 (0·05 M.), nicotinamide (0·02 M.), ATP (0·003 M.), $MgCl_2$ *(0·01 M.), substrate as indicated, and 2·5 ml. enzyme suspension in a total volume of 6 ml.*

Substrate	Citrate formed in 2 hrs. μM.
Oxalacetate (0·03 M.)	0·5
Oxalacetate (0·03 M.)+Acetate (0·05 M.)	0·7
Oxalacetate (0·03 M.)+Pyruvate (0·05 M.)	1·05
l-Malate (0·05 M.)+Pyruvate (0·05 M.)	0·2
None	0·0

The formation of citrate was also demonstrated by paper chromatography. The acids were removed from the reaction mixture with Amberlite resin IRA 400, and eluted with 80 per cent. formic acid. The solution was concentrated on a steam-bath and applied to the filter-paper (Fig. 5).

(8) *Formation of 'Active' Acetate*

Although it was possible to demonstrate the utilization of pyruvate (Table II), attempts to demonstrate an 'active' acetate have failed. There was no indication of acetyl phosphate or acetyl-co-enzyme A formation from either pyruvate or acetate, when tested by the hydroxamic method of Lipmann and Tuttle (1945).

DISCUSSION

Under the microscope the active material appears to consist of spherical particles, which are approximately 1μ in diameter, and are stained with Janus Green B. Millerd *et al.* (1951) consider the staining reaction with Janus Green B to be a unique property of intact mitochondria. However, the specificity of this stain for plant mitochondria has not been fully established (Newcomer, 1951; Stafford, 1951).

The experimental evidence supports the view that the particulate fraction catalyses the reactions of the Krebs cycle. However, there is insufficient evidence to establish the spherical particles as the seat of the Krebs cycle enzymes. It was not possible to fulfil the criteria which Hogeboom and Schneider (1950) consider should be satisfied before a biochemical property is ascribed to a structural element.

The enzyme system requires Mg ions and ATP for maximum activity. According to Green (1951), there are some indications that the power to carry on oxidative phosphorylation and the requirement for ATP run parallel. It is of some interest that particulate material isolated from water homogenates of pea seedlings required cytochrome *c* for oxidative activity and was not activated by ATP (Meeuse, 1952), whereas material isolated from a buffered sucrose homogenate of mung bean seedlings has been shown to carry out oxidative phosphorylation (Millerd *et al.*, 1951). Although malic acid is oxidized by the enzyme system, the addition of DPN produces an increased oxygen uptake with a corresponding accumulation of keto acids. When DPN is added to the reaction mixture enzyme+malate+acetate, the accumulation of keto acids is greater than that calculated from the increased oxygen consumption. The co-enzyme induced accumulation of keto acids is reduced by the addition of succinate or *iso*citrate to the reaction mixture. Addition of DPN to the reaction mixture enzyme+malate+pyruvate does not increase oxygen uptake.

An explanation of these results may be put forward on the assumption that the rate of oxygen uptake is limited by the concentration of the terminal oxidase, or by some other factor in the electron path from substrate to oxygen. Since the addition of cytochrome *c* does not affect the rate of oxidation, the most likely limiting factor would seem to be cytochrome oxidase or possibly Slater's factor (Slater, 1950). The affect of adding DPN is to increase the rate of the reaction:

$$\text{Malate}+\text{DPN} \rightleftharpoons \text{Oxalacetate}+\text{DPN.H}_2.$$

In the presence of DPN the rate of this reaction will depend on the concentration of malic-dehydrogenase, and on the rate of oxidation of reduced co-enzyme. With malate+acetate as substrates we may suppose that intermediates of the Krebs cycle are formed which compete with malic acid for the terminal oxidase. The addition of DPN to the system enzyme+malate+acetate would be expected to increase the rate of oxidation of *l*-malate and saturate the terminal oxidase with electrons, thus inhibiting the oxidation of other substrates. This situation would lead to an accumulation of keto acids without a corresponding increase in oxygen consumption; which is in agreement with the experimental findings. Further, by increasing the concentration of succinate or *iso*citrate the activity of their dehydrogenase systems should be increased. Consequently these dehydrogenase systems would be expected to compete effectively with malic-dehydrogenase and so reduce the formation of oxalacetic acid—which again is in agreement with the experimental results.

This explanation requires that the malic oxidase of the system be partly dissociated. Huennekens (1951) has demonstrated that whereas the dissociated malic oxidase is inhibited by oxalacetate and pyruvate, the conjugated pyridinoprotein is not inhibited. Under anaerobic conditions and with methylene blue as an electron acceptor, inhibition of malic-dehydrogenase is observed (Table V). Under aerobic conditions considerable quantities of keto acids are formed without any apparent inhibition of malic oxidase (Fig. 3). No explanation for this discrepancy has been found.

DPN did not increase the accumulation of ketoglutarate when supplied to the reaction mixture enzyme+*iso*-citrate at pH 7. However, at pH 9 there was a marked accumulation of α-ketoglutarate (Table IV). *Iso*citric dehydrogenase is usually considered to be TPN specific (Vennesland and Conn, 1952). The observed stimulation of *iso*citric-dehydrogenase by DPN may be explained in at least two ways. The preparation may contain an enzyme system capable of converting DPN to TPN in the presence of ATP. Such a system has been reported in pigeon liver preparations by Mehler, Kornberg, Grisolia, and Ochoa (1948). Recently Kornberg and Pricer (1951) have demonstrated the presence of two distinct *iso*citric-dehydrogenases in yeast. One of these was TPN specific, the other was DPN specific and oxidatively decarboxylated *iso*citrate to α-ketoglutarate without oxalosuccinate serving as an intermediate. It is possible that the preparation contains a similar enzyme. Should this be true there exists an interesting parallel between the two *iso*citric-dehydrogenases and malic-dehydrogenase and the 'malic' enzyme of Ochoa.

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The Effects of Thiamin and Nicotinic Acid on Meristematic Activity in Pea Roots

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SUMMARY

The results of experiments with isolated pea roots are presented which show the effects on growth of providing nicotinic acid and thiamin in the medium. Certain of the experiments involve two experimental variables, the presence and absence of vitamins in the medium and tip inocula of different lengths. Growth is analysed in terms of overall length increase, increment in number of cells, rates of division, and mature cell volume. It is shown that both vitamins affect division, and in the case of vitamin B_1, through a primary effect which may be either on mature or meristematic tissue. Evidence has not been obtained that with the material of this investigation the vitamins have any effect on the growth of the cell. Further evidence is provided in favour of the hypothesis that division in the meristem depends on products formed in the meristem, and on a different set of metabolites synthesized in mature tissue.

INTRODUCTION

THE subject of this investigation is the effect of nicotinic acid and thiamin on cell growth and cell division in pea roots. The problem has previously been examined by Addicott (1939, 1941), who compared the anatomical features of roots that had been grown in vitamin deficient and complete media and was able to show that in deficient roots the meristem is small and the frequency of mitotic figures low. He also showed that in deficient roots the length of mature cells is only 60 per cent. of that in roots cultured with a complete medium. It was concluded that both vitamin B_1 and nicotinic acid affect division as well as extension. It is an important feature of the work of Addicott, however, that the observations were made with roots from the third and fourth subcultures after deficient conditions had been established.

The subject has been reinvestigated for two reasons. A different technique for the analysis of meristematic activity is now available and recent results suggest that it may be necessary to interpret events in the meristem in terms of primary effects established in other parts of the root. The investigation is designed to show the effects of deficiencies on cell division and cell growth and to determine the extent to which the changes observed are due to primary effects in the growing region. The nature of the tissue in which the primary reaction to the vitamin occurs is clearly an important aspect of the situation. We have shown elsewhere (Brown and Wightman, 1952) that meristematic activity may be influenced by a supply of metabolites from mature tissue, and this suggests the possibility that the effect of the vitamins is established in

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ature tissue and that the promotion of division is a consequence of a stimu- tion of a flow of metabolites to the apex.

The observations of this investigation have been made with pea roots grow- g in isolated sterile culture and they have all been made with roots in the rst subculture. Roots grown from tips excised from germinated seeds cannot e used since these do not react to vitamins in the medium. We have found, as onner and Addicott (1937) did with thiamin and as Addicott and Devirian id with nicotinic acid, that it is only after the first transfer that the vitamin ontent of the root is reduced to a sufficiently low level to give a reaction to an xternal vitamin supply.

The general experimental technique used is similar to that described in an arlier paper (Brown and Wightman, 1952). Samples have been taken at ntervals of 24 hours from a group of cultures and on the roots of each sample he lengths, breadths, total number of cells, and the number of meristematic ells have been determined. From these data the effects of the vitamins in erms of overall length increase, cell number increase, rates of division, and ell growth may be analysed. The general nature of the stimulation has been nvestigated by comparing the effects of deficient and complete media with ip inocula of different lengths. Clearly if the primary effect is established in nature tissue, then there should be no immediate response to vitamin supple- nent when the tip is of such a length that it carries only meristematic and ome expanding cells.

MATERIALS AND METHODS

The experimental material used in this investigation is taken from sub-cultures from roots that have been derived from tips excised from germinated seeds. The parent roots for the subcultures are developed by the technique described in an earlier paper (Brown and Wightman, 1952). At the end of one week of growth the parent roots are 60–70 mm. in length and are still growing vigorously, although they are then in a medium that contains no vitamins. On the seventh day the tips of these parent roots are excised, the length of the tips being adjusted by aligning the roots against scales marked on the lower surfaces of the culture dishes. Each tip is transferred to 15 ml. of liquid medium in a Petri dish, two tips being placed in each dish, and the culture is transferred to an incubator maintained at 25° C. For each experiment 40–50 dishes are set up and this number, allowing for failures due to chance infection, provides for 6 or 7 samples of 5 dishes each. On each day of the experimental period a random sample is withdrawn which yields 10 roots.

In some series the complete nutrient medium recommended by Addicott and Devirian (1939) has been used. This contains sucrose, an inorganic salt mixture, thiamin, and nicotinic acid. Nicotinic acid is supplied at the rate of 0·5 mg./litre and thiamin at the rate of 0·1 mg./litre. The effects of higher concentrations of the vitamins have been examined, and it has been found that they do not promote enhanced growth rates. All solutions have been sterilized by autoclaving.

The measurements that are made on each sample are similar to tho described in an earlier paper. In certain cases the length of each root measured, and then the total number of cells and the number of meristemat cells are determined in the whole sample. When estimates of cell volume a required each root is first cut into an apical and a basal fragment. The apic fragment is always 1·0 cm. in length and after cutting the length of the bas portion is measured. Three measurements of breadth are also made on t basal fragment, one at the apical, one at the basal end, and one in the centr All the apical and all the basal fragments from a single sample are assembl separately and determinations of total and meristematic numbers of cells a made on each group separately. The procedures used in the determinations length and numbers of cells are described elsewhere (Brown and Wightma 1952). The measurements of breadth are made microscopically against calibrated micrometer eyepiece.

From the length measurements and from the determinations of total ar meristematic numbers of cells, averages for each root are calculated. From tl cell number data rates of division are obtained. From the breadth measur ments a mean breadth of the basal fragment is estimated, and this with tl mean length gives a mean volume. This quantity when divided by the numb of cells in the basal fragment gives an average value for cell volume. The cel in the basal fragment are all mature cells, and the average volume therefo represents the final size of the cell after growth has occurred.

Two sets of variables have been used in the investigation: the presence absence in the medium of nicotinic acid or thiamin, and various lengths of t inocula. Three tip lengths have been used, 5·0, 10·0, and 20·0 mm. All thr carry 40,000–50,000 non-vacuolated meristematic cells. As the length in creases the number of vacuolated cells also increases; the 5·0-mm. tip carri 45,000 vacuolated cells; the 10·0-mm. tip 65,000, and the 20·0-mm. ti 105,000. A similar experimental provision has already been made with firs week roots. In this case, however, the tip lengths have been 3·0, 6·0, an 10·0 mm. The differences in length between the two series are the result different structural characteristics in the parent roots. First-week cultures ar established from seedling roots in which the meristems are larger and tl numbers of longitudinal ranks of cells in mature regions greater than they ar in roots cultured in isolation.

EXPERIMENTAL RESULTS

The results of two groups of experiments are given below. The first has bee designed to show the effects with a constant tip length of the provision in th medium of each of the vitamins separately; and the second the effects different tip lengths in the presence and absence of both substances in th medium.

With each group the length, total cell number, the meristematic ce number data, and the rates of division are considered separately and in tha order.

Group I. In this group the effects of the following media are compared: a ll medium, one containing nicotinic acid only, another containing thiamin nly, and a fourth lacking both vitamins. The observations have all been ade with 10·0-mm. tips and the experiments have all been continued for days.

The length data for this group are given in Table I.

TABLE I

ength (mm.) with media lacking both vitamins (−N−B), containing both +N+B), containing nicotinic acid only (+N), and containing thiamin only (+B). Time in days

Time	*−N−B*	*+N*	*+B*	*+N+B*
0	10·0	10·0	10·0	10·0
1	16·0	16·0	16·0	16·5
2	18·0	20·0	22·0	22·0
3	23·0	25·0	29·0	29·0
4	19·0	29·0	33·0	35·0
5	17·5	33·0	41·5	46·0
6	20·5	37·0	46·0	58·0
7	22·0	39·0	52·0	63·5

In the absence of both vitamins, length growth is limited, the final length eing 22 mm. Nicotinic acid alone has a considerable effect, giving a final ength of 39 mm. Thiamin alone, however, has a still greater effect, the final ength in this case being 52·0 mm. The greatest effect is given with the two vitamins supplied together, the final length in this case being 63·5 mm. It may be noted, however, that whereas the effects of thiamin and nicotinic acid when provided alone are evident after the first day, the effect of nicotinic acid in the presence of thiamin is only developed after the fourth day. Clearly the

TABLE II

Total cell numbers and non-vacuolated cell numbers (in thousands) with different media indicated by symbols as in Table I

	Time	*−N−B*	*+N*	*+B*	*+N+B*
Total	0	100	100	100	100
	1	120	115	120	130
	2	130	140	138	145
	3	150	153	160	175
	4	107	175	185	205
	5	133	190	214	245
	6	135	220	250	305
	7	155	230	306	335
Non-vacuolate	0	50	50	50	50
	1	41	41	37	45
	2	35	40	39	41
	3	23	31	33	37
	4	18	37	27	37
	5	15	38	35	40
	6	11	38	44	49
	7	10	34	50	55

effect of nicotinic acid in the presence of thiamin is limited. It is probable tha in all series length increase continues throughout the 7 days. The position i doubtful in the absence of both vitamins, but if the value for the third day i an abnormally high one, then it may be that length continues to increas throughout the experimental period in this series also.

The total cell number data of Group I are given in Table II. In the absenc of both vitamins there is a limited increase until the third day, and number then probably remain more or less constant. With nicotinic acid alone cel numbers increase steadily with time and reach a final value greater than in th absence of both vitamins. With thiamin alone the increase is greater than wit nicotinic acid alone. The greatest increase is given with both vitamins. At th same time the difference between this and the increase with thiamin only i not great. The results suggest that nicotinic acid has a greater relative effec when supplied alone than with thiamin. The data further indicate that in the two series provided with thiamin the rate of increase tends to accelerate wit time.

The non-vacuolated cell number data of Group I are also given in Table II (lower half).

When neither vitamin is supplied in the medium the number of non-vacuolated cells decreases rapidly with time. The number tends to remain more or less constant, however, when the medium contains either one or both vitamins. It is significant that although the numbers of cells formed and the rates of division in the three vitamin-supplied series are different, nevertheless the numbers of non-vacuolated cells in these are all more or less the same.

The rates of division for the experiments of Group I are given in Table III.

TABLE III

Rates of division (number of divisions meristematic cell/24 hours) with differen media. Symbols as in Table I

Time	−N−B	+N	+B	+N+B
1	0·4	0·3	0·5	0·6
2	0·3	0·6	0·5	0·4
3	0·7	0·4	0·6	0·8
4	—	0·7	0·8	0·8
5	—	0·4	0·9	1·1
6	—	0·8	1·0	1·3
7	—	0·3	1·3	0·6

When no vitamin is supplied there is no increase in cell numbers after the third day, and in this series therefore no rates are given for the final perio after the third day. The series supplied with nicotinic acid only gives the lowest rates of the three vitamin series. Also the values for this series do no show any consistent trend. The two series supplied with thiamin give values o about the same order, although that provided with both vitamins may give the slightly higher rates. In both series supplied with thiamin the rate tends t crease with time, and in that with both vitamins it reaches a peak on the xth day and then falls.

Group II. In this group of experiments the effect of a medium containing o vitamins and of one containing both vitamins have been compared with p inocula of 5·0, 10·0, and 20·0 mm. in length. The experiments in this group ave been continued for 6 days.

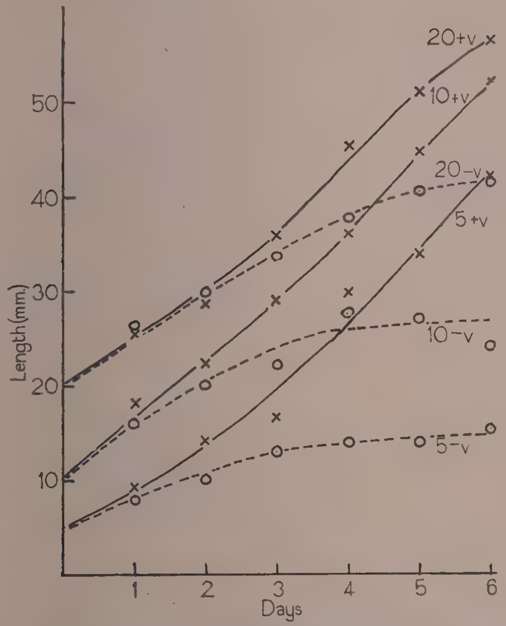

FIG. 1. Change in length with time of roots started from 5·0, 10·0, and 20·0 mm. tips, with a full medium (+*V*), and with a medium deficient in both vitamins (−*V*).

Two series in this group of experiments involve the same treatments as two in the previous group. In both groups there are series from 10-mm. tips in a full and in a completely deficient medium. Thus two duplicate sets of results are available which provide indications of the errors of the techniques used.

The length data for the experiments of this group are given in Fig. 1.

The 10·0-mm. tips from Group I and II give values, at 6 days, with deficient media, of 20·5 and 24 mm. and, with full media, of 58 and 52 mm.

In each case length increase is limited in the deficient medium. At the same time the increment with this medium increases with the length of the original tip. Further it is probable that, with all tip lengths in the absence of vitamins, length increase continues throughout the experimental period.

When the vitamin supplement is provided, growth is vigorous whatever the tip length. The final length of course varies with the length of the original tip, but the course of growth is evidently the same in all cases. Fig. 1 shows tha the relevant curves all follow approximately the same course.

The total cell number data for the experiments of Group II are given i Fig. 2, and are shown also in Table IV.

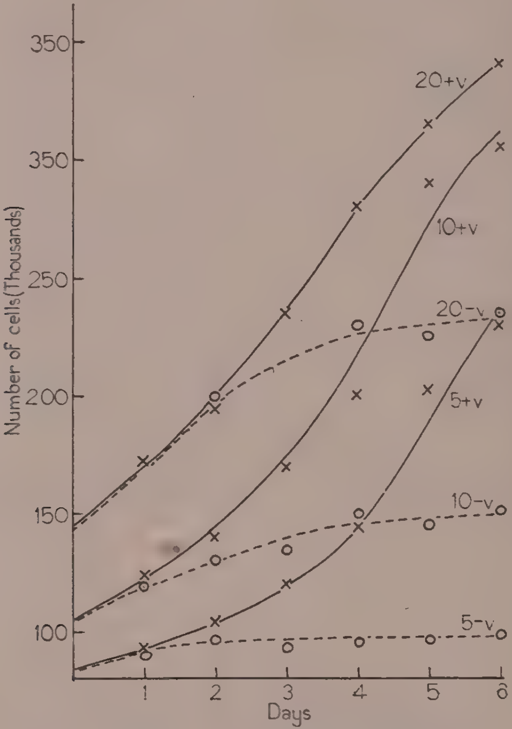

FIG. 2. Change in total number of cells with time in roots started from 5·0, 10·0, and 20·0 mm. tips with a full medium (+*V*), and with a medium deficient in both vitamins (−*V*).

The series from 10·0-mm. tips from Groups I and II give values, at 6 day with deficient media, of 135,000 and 151,000 respectively, and with full medi of 305,000 in both cases.

When vitamins are not supplied the cell number increment is limited whatever the length of tip. As with length, however, with deficient media the cel increment tends to increase with increasing length of tip. The increase, however, does not continue throughout the experimental period. With the 5·0-mm. tip it ceases on the second day, with the 10·0-mm. tip it ceases on th fourth, and with the 20-mm. tip again probably on the fourth day.

When vitamin supplement is provided, increase in cell numbers continue throughout the experimental period. With the 5·0- and the 10·0-mm. tip, cel umbers are greater at all corresponding stages when vitamin is supplied. It is gnificant, however, that with the 20·0-mm. tip, cell numbers are only greater n the presence of vitamins after about the third day. The results suggest that hereas response to the vitamins is immediate with the shortest tip it is deyed with the largest. Finally it may be noted that, when the medium contains oth vitamins, although the final cell number varies with the length of the riginal tip, the course of change of cell number with time is not the same in all eries. Fig. 2 shows that whereas the relevant curve for the 20·0-mm. tip ends to the linear form, that for the 5·0-mm. tip is convex to the horizontal xis. The data given by the 10·0-mm. curve are similar to those of Table I nd in general follow the same course as the 5·0-mm. curve, although the ise from the origin is steeper. Clearly when the tip is short there is an nitial lag phase in cell-number increase, and this is followed by one of ccelerated increase. As the tip gets longer the lag phase becomes shorter and ess intense, and the rate of increase over the whole period tends to become more or less constant.

The data for numbers of non-vacuolated cells for the experiments of Group II are given in Table IV.

TABLE IV

Numbers of non-vacuolated cells (thousands) in tips of different initial lengths with different media. Total cell numbers are shown in brackets. Media containing both vitamins (+V), neither vitamin (−V)

Time days	5·0 mm.		10·0 mm.		20·0 mm.	
	+*V*	−*V*	+*V*	−*V*	+*V*	−*V*
0	40 (85)	40 (85)	40 (105)	40 (105)	40 (145)	40 (145)
1	25 (91)	26 (90)	30 (124)	31 (120)	39 (172)	38 (170)
2	21 (105)	20 (96)	26 (140)	26 (130)	37 (195)	34 (200)
3	25 (120)	20 (95)	36 (170)	24 (135)	40 (235)	33 (220)
4	31 (145)	20 (95)	37 (199)	20 (150)	38 (280)	26 (230)
5	36 (202)	16 (96)	40 (275)	17 (145)	44 (315)	22 (225)
6	43 (230)	11 (98)	45 (305)	13 (151)	50 (340)	19 (234)

The series from 10·0-mm. tips from Groups I and II give values at 6 days with deficient media of 11,000 and 13,000 respectively and with full media of 49,000 and 45,000 respectively.

Whatever the length of the initial tip, the number of meristematic cells is lower in the absence than it is in the presence of vitamins. In a deficient medium the number of non-vacuolated cells always decreases with time. The decrease, however, is greatest with the shortest tip and least with the longest. When a full vitamin supplement is provided with the longest tip the numbers of non-vacuolated cells remain more or less constant throughout the whole experimental period; with the shortest they tend to decrease at first and then to increase. The 10·0-mm. tip occupies an intermediate position in this as in other respects.

The rates of division for the experiments of Group II are given in Table V.

TABLE V

Rates of division (division/meristematic cell/24 hours) with different tip lengths and different media. Symbols as in Table IV)

Time (days)	5·0 mm.		10·0 mm.		20·0 mm.	
	$+V$	$-V$	$+V$	$-V$	$+V$	$-V$
1	0·2	0·2	0·5	0·4	0·7	0·6
2	0·6	0·3	0·6	0·4	0·6	0·8
3	0·7	—	1·0	0·2	1·0	0·6
4	0·9	—	0·8	0·7	1·2	0·3
5	1·7	—	2·0	—	0·9	—
6	0·7	—	0·7	—	0·5	—

The values from Groups I and II for full media are clearly very similar. The peak value in Group I, however, is given on the sixth day, whereas in Group II it is given on the fifth; and whereas it is 1·3 in Group I, it is 2·0 in Group II.

Whatever the tip length, while division is proceeding in the absence of vitamin supplement, the rate is lower in the absence than it is in the presence of vitamin, except over the first 2 days with the longest tip when the rates with the two treatments are more or less the same. In all series provided with vitamins the rate tends to be low at first, to increase with time, to reach a peak value, and then decrease. The initial rate, however, tends to increase with increasing length of tip. The peak value is lowest and is reached earliest with the longest tip. The change in rate is clearly greatest with the shortest tip and least with the longest.

DISCUSSION

The data presented above may be considered in two connexions: (1) in relation to the general characteristics of growth in roots taken from subcultures, and (2) in relation to the growth effects of the vitamins.

In an earlier paper (Brown and Wightman, 1952) we have presented data showing that, in first-week roots, meristematic activity is determined by two sets of metabolites generated in the tip and in mature regions respectively. This conclusion is based on the observations that, with respect to increase in total cell numbers, there is an initial lag phase with short-tip inocula which tends to get shorter the longer the tip; and with respect to the rate of division when the tip is short, the rate increases with time, reaches a peak value, and then falls, the peak rate tending to be greater the shorter the tip and tending to occur later the shorter the tip. With a full medium all these conditions are shown by the data of Tables IV and V. The results of the present series of experiments confirm the earlier and to that extent provide further evidence in favour of the hypothesis outlined.

The results of the earlier series of experiments showed that length increase is not a simple function of change in the absolute number of cells. This feature is shown by the data of Figs. 1 and 2. With a full medium, length increase follows the same course with time whatever the length of the tip ioculum. With the same medium, however, with the short tip, increase in umbers of cells shows an initial lag phase. Clearly, in this phase, length increase is being partly determined by the expansion of non-vacuolated cells carried over from the parent root. This conclusion is consistent with the results o Table IV, which show that with the short tip with a full medium there is an itial decrease in the number of non-vacuolated cells. Again it is evident that he increase in length of roots from short tips in deficient media is not related o a corresponding increase in cell numbers. The data of Fig. 1 show that in days there may be a threefold increase in the length of these roots, whereas e data of Fig. 2 show that within the same period there is little or no increase cell numbers. At the same time it is evident from Table IV that there is a arp decrease in the number of non-vacuolated cells in this series. Clearly e increase in length in this case also is due to the vacuolation of meristematic lls carried over from the parent root.

The continued expansion of non-vacuolated cells in the absence of division mphasizes an important aspect of the meristem. This tissue is not a structural ntity, but evidently a highly labile zone. In general it seems that when ivision is slow the size of the meristem tends to decrease. The results ssembled here and in the earlier paper suggest that the time that elapses etween the formation of a cell and the development of a well-defined acuole is relatively long and is not affected by the rate of division. Thus when ivision is slow the number of cells that pass out of the non-vacuolated state is elatively greater than the number that is formed and as a result the overall ize of the meristem decreases. This is evidently the position with the short tip n the early stages of culture in a full medium. In the later stages of culture he rate of division increases the number of cells formed relative to the number hat pass out of the non-vacuolated state. The consequence of this is that he overall size of the meristem increases as shown by the data of Table IV. The size of the meristem, however, does not depend only on the rate of division. There is some evidence that nutritional conditions may affect the rate at which cells traverse the phase between formation and vacuolation. As indicated above, although the rate of division is lower with nicotinic acid alone than it is with thiamin alone, nevertheless the number of non-vacuolated cells with the two treatments is about the same (Table II). Clearly nicotinic acid may have an effect in maintaining the non-vacuolated state and thus in prolonging the period between formation and vacuolation. A similar phenomenon has been observed with tips excised from germinated seeds. When these are transferred to a simple sugar solution, a solution of inorganic salts, or water, division is limited and similar in extent with all three treatments. The effects of the different solutions on vacuolation, however, are different. With sugar solution and water, vacuolation is rapid, but with inorganic salts alone it is comparatively slow.

The data of Table II show that the two vitamins have profound effects on meristematic activity. In the presence of one or other of the vitamins or of the two together, the number of cells formed is large, the rate of division is high, and the meristem remains large. In the absence of both vitamins division is restricted and the meristem shrinks. Clearly the stimulating effects of the two vitamins on length increase (Tables I and III) must be attributed primarily to corresponding effects on division. At the same time the possibility remains that greater overall length may be partly a result of enhanced cell growth. This possibility has been examined by determining, by the technique described earlier, the mature cell volume in each sample of the first group of experiments The results are shown in Table VI.

TABLE VI

Mature cell volumes (*ml.* × *10*$^{-8}$) *in roots cultured with different vitamins. Symbols as in Table I*

Time	$-N-B$	$+N$	$+B$	$+N+B$
1	8·7	10·8	11·5	10·1
2	8·2	8·0	10·1	8·5
3	8·5	9·9	9·3	8·2
4	8·2	9·3	9·3	7·3
5	9·9	10·8	11·5	11·2
6	8·8	10·3	8·8	8·1
7	13·8	9·0	10·0	9·3
Mean	9·4	9·7	10·1	9·0

It is clear from the data of Table VI that at least with the roots used in the experiments of this investigation vitamin treatment has had little or no effec on mature cell size. A comparison of the data of Figs. 1 and 2 also suggests that vitamin supply does not affect extension. In the absence of vitamins increase in cell numbers with the 5·0 tip ceases on the second day and with the 10·0- and 20·0-mm. tips on the fourth day. Length increase, on the other hand, probably continues throughout the experimental period with all tip lengths. Evidently the reduction of the vitamin content to the level at which division is arrested does not have an effect on cell extension.

It is clear from the data of the first group of experiments that thiamin has a more pronounced effect on division than has nicotinic acid. The number of cells and the rate of division are considerably greater when thiamin is given alone than when nicotinic acid only is provided. Further, the data suggest tha in the conditions of the present series of experiments, and with respect to division, nicotinic acid may act as a substitute for thiamin. It is significant tha whereas nicotinic acid has a considerable effect when supplied alone, it ha only a slight affect in the presence of thiamin. The difference between the deficient and the nicotinic acid series (Tables II and III) is comparatively large while between the thiamin and thiamin+nicotinic acid series it i relatively small. This conclusion does not carry the implication either tha metabolically the functions of the two substances are the same or that nicotini acid can be substituted for thiamin at all stages of growth. There is abundan evidence that metabolically the two substances are involved in differen systems, and Addicott and Devirian (1939) have shown that after several sub cultures growth in the presence of thiamin and in the absence of nicotini acid is very restricted.

Since nicotinic acid gives only a slight stimulation in the presence of hiamin, it is evident that the results of the second group of experiments must e interpreted primarily in terms of the effect of thiamin. It is clear from the urves of Figs. 1 and 2 that the stimulation of division due to thiamin may in certain cases be attributed to a direct effect of this substance on the metabolic ctivity of the meristem itself. With short tips (5·0 and 10·0 mm.) in which there re relatively few mature cells there is an immediate response to the provision f vitamins. Even after 24 hours, roots in a full medium have a larger number f cells than those in a deficient medium.

On the other hand, with the longest tip (20·0 mm.) the position is more complicated. With this material there is no response until after the second day. On the second day there is little difference between roots from deficient and ull media with respect to the total number of cells they contain. All the nocula, however, are derived from first-week roots cultured in the same conditions. The meristems of all the inocula are therefore in the same state. f the meristems of short tips are vitamin deficient, those of long tips are undoubtedly so too, and long tips might be expected to respond to vitamin upplement in the same way as short tips. In this connexion the conditions of growth in the parent root are relevant. First-week material is grown in a medium which contains no vitamins, and it continues to grow vigorously in his medium for at least 9 days. The subcultures are made at 7 days, and the conditions necessary for the growth of these show that when short tips are used they are vitamin deficient. Thus the first-week root continues to grow vigorously for at least 2 days while there is little or no vitamin in the tip. Clearly it is probable that growth is being maintained through the activity of vitamin-containing tissues in mature parts of the root. It is probable that vitamin-rich tissues produce certain metabolites which are subsequently transferred to the tip and there promote division. This interpretation suggests that the reaction of long tips is due to a vitamin gradient along them. The excision of the long tip involves removing with the meristem tissue which carries thiamin. When this tip is placed in a full medium the requirements of the meristem are already being met by metabolites produced in vitamin containing mature tissue.

Thus the present series of results show that while the growth effect of thiamin is due to a stimulation of division, this stimulation depends on the production of one or possibly a complex of metabolites. The incorporation of thiamin into the particular system that promotes the synthesis of the division stimulants may occur either in mature or meristematic tissue. Conditions may arise, however, in which thiamin is present only in mature tissue, in which case the appropriate substances are produced in this tissue, transported to the meristem, and there promote division.

The present series of results are consistent with those of Addicott (1941) in showing that in the absence of thiamin and nicotinic acid division ceases and the meristem contracts. In this series of experiments, however, evidence for an effect of these substances on cell growth has not been obtained. At the same time it may be emphasized that the roots with which Addicott worked were from the third and fourth subcultures after transfer to deficient media whereas those of this investigation have been from the first. After repeated subculturing the vitamin content of the root is likely to be lower than it is in the first subculture, and it is possible that the different results obtained in the two investigations with respect to cell extension is a consequence of different vitamin contents. It is possible that the effect on extension is given at lower vitamin levels than the effect on division, and it may be that when roots from the first subculture are used the internal vitamin content has been reduced to a level sufficiently low to show the division effect but not low enough to show the extension effect.

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The Absorption of Potassium by Cells in the Apex of the Root

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SUMMARY

The purpose of this investigation is the study of the properties of the cell with regard to potassium absorption as the cell expands. Serial sections each 1·5 mm. in length have been cut along the course of maize roots over the first 9·0 mm. from the tip. The sections have been immersed in 0·01 N. solution of potassium chloride in water and in 2 per cent. fructose. Absorption from these solutions has been measured over a period of 48–72 hours. It is shown that absorption by the first section which consists almost entirely of meristematic cells is abnormally low, and this is attributed to the absence of a tonoplast surrounding a central vacuole in the cells of this section. The different initial rates with the different sections indicate that after vacuolation in the root has occurred the rate per unit area of surface decreases as the cell expands. When the data are reduced to a unit cell basis, however, they show that, per cell, absorption increases as expansion occurs in the root. This is attributed to an increasing protein content, and the decrease in rate per unit area to a corresponding decrease in protein per unit area. At the same time it is shown that during cell growth in the root the cytoplasm differentiates in such a way that absorption per unit protein increases.

After excision, whether growth occurs or not, the protein content does not increase. The final internal concentrations in the different sections vary according to the growth that occurs. When vacuolation has occurred and growth is limited the final internal concentration is greater than it is at the beginning of the experiment. When growth occurs, on the other hand, the final internal concentration may be lower. This is taken to indicate that absorption depends on an inward secretion into the vacuole which is independent of surface area and an outward diffusion into the medium which increases with increase in surface area. It is suggested that the results obtained with sugar support this interpretation. When sugar is provided the rate of absorption is always stimulated and when growth does not occur the final internal concentration is enhanced. When growth occurs, however, sugar not only stimulates absorption but also the expansion of the cell, and the latter effect leads to a final concentration which may be lower than that given in the absence of sugar.

INTRODUCTION

THE present group of observations have been designed to elucidate the conditions that determine changes in salt absorption as the cell expands. Most studies on the absorption of inorganic ions have been made with slices from storage tissues, with mature giant cells such as those of *Valonia* and *Halicystis*, or with intact organs such as roots which may or may not grow during the experimental period. Studies with slices and giant cells provide data on

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changes promoted in a relatively stable system by externally applied variables. Studies with organs provide similar data, although with these, when growth occurs, the position may be complicated by the induction of new cells which act as further absorption units. A different experimental approach is proposed here, however, which may yield data of a different type. If observations can be made on expanding cells the characteristics of which can be defined, then the effects of biological variables in the situation may be analysed. An attempt has been made to provide a preliminary analysis of this type. The data provide indications of the effects of surface area, of the presence of the vacuole, of vacuolar volume, and of the mass and differentiation of the cytoplasm on the absorption process. The observations are not comprehensive, but they do suggest that types of data may be assembled by the use of growing cells which may not be accessible through the use of more traditional experimental objects. The results that have been assembled are also of some significance in two other connexions. They show how the properties of the cell with regard to salt absorption change as expansion occurs, and they are therefore of immediate relevance to an elucidation of the factors that control the growth of the cell. Further, since the observations have been made with root tissue they are of some importance in relation to the conditions that determine absorption in the root as a whole.

The effects of the biological variables have been examined in relation to the absorption of potassium, the choice of this ion being due to the fact that it is intimately involved in the expansion of excised tissue (Brown and Sutcliffe, 1950). The general technique of the investigation involves cutting serial sections along the course of the root from the tip to the limit of the growth zone, suspending groups of corresponding sections in salt solutions, and measuring the changes with time in the concentration of the solute in the external medium. In certain connexions the results are related to the number of cells each section contains. The successive sections incorporate cells at progressively advanced stages of development. The values given by the different sections immediately after excision, therefore, show how salt absorption is affected by the developmental changes that occur when the cell grows in the intact root. The experiments are continued for 48 to 72 hours, and during this time certain of the sections may increase considerably in volume. This increase in all sections except the first is entirely by cell expansion and does not depend on division. Thus the values given in the later and final phases of the experimental period show how absorption changes when expansion occurs in isolation from the parent root. The experimental design has been elaborated to show the effect of sugar on absorption. Sugar promotes the growth of excised fragments, and the primary purpose in using this variable is not the examination of the effect of different metabolic states but of the effect on absorption of different degrees of expansion of the same tissue.

It is shown below that the characteristics of the absorption process change profoundly as the cell expands, and an attempt has been made to relate these ot only to changes in volume and surface area but also to internal concentraon and protein content. The internal concentration of the solute has the me significance in this as it has in any other tissue. The protein content, owever, has a particular importance in growing tissue. The volume of the rotoplast relative to the other components of the cell varies, and some easure of the relative amount of cytoplasmic material is therefore required. he protein content is not an entirely reliable measure of this quantity, but is probably the most satisfactory available. It certainly provides a more eliable estimate than any metabolic standard such as respiration since it has een shown that the metabolic pattern of the cell changes vary considerably uring the course of growth (Robinson and Brown, 1952).

The concentration data given below are calculated from estimates of the olume and the absolute potassium content. It is recognized that this can only e a very approximate index of the situation. The volume estimate is crude, specially with the first section, which is conical. Also the proportion of the hole volume occupied by water probably varies. In the apical section there re relatively few vacuoles and the intercellular space system becomes more xtensive as the tissue matures. Further, there is no evidence that the proporon of potassium in the tissue in a free diffusible form is constant. At the ame time, since at least 80 per cent. of the volume of all sections is probably ccupied by water and since, when vacuoles are present, most of the potasium is probably in free solution in the vacuolar sap, large differences in mounts per unit volume probably represent corresponding differences in oncentration. As the values given can only have limited significance, they re presented in terms of mg./ml. and not in terms of molar quantities.

The results given below are interpreted in terms of a system which involves n inward secretion and a simultaneous outward diffusion of solute. It is ssumed that the measured absorption represents the difference between these wo processes and that at the stage when absorption ceases a steady state has een reached at which the rates of the two processes are the same. The terms hat are used below have been framed in relation to this system of interpretaion. Absorption is the measured uptake and is understood to represent the ifference between the inward and outward flow over the whole surface of he cell or fragment. Inflow represents the transfer of solute across the whole urface area from the medium in unit time, and outflow the diffusion into the medium across the whole surface area in unit time. Absorption, inflow, and outflow are descriptive terms which as terms carry no implications with regard to surface area or concentration gradients. Permeability is used in the conventional sense of the quantity of solute that traverses unit area along unit concentration gradient in unit time.

The scheme of interpretation used here is suggested by the data. As shown below, an increase in surface area may in certain circumstances be accompanied by a decrease in final internal concentration. Further, the evidence indicates that the rate of inflow does not decrease as the saturation content is approached. These two sets of observations suggest the operation of an outflow while the inflow is proceeding. The demonstration of a low permeability in the cytoplasm to electrolytes may not affect the position. Overstreet and Broyer (1940) have shown with intact roots of barley that the exchange of radioactive and stable isotopes across the protoplast surface may be very restricted. Some, however, apparently does occur and an exchange measured in hours may be significant for a process such as that of this series of experiments which occupies days.

In the tissues of these experiments the outflow may have certain exceptional characteristics. The outflow may involve a discharge into xylem elements since these are fully developed in at least some of the sections of the series. Clearly the possible occurrence of a 'leak' into the xylem does not invalidate the general concept of an outflow since it may be considered as one aspect of it. At the same time it must be recognized that the leak into the xylem may not conform to the conditions of diffusive flow, and may therefore not conform to the general character of the outflow postulated above.

The interpretation of the absorption process must depend on a consideration of the simultaneous growth of the cells or tissue. The interpretation requires two sets of data: on the state of the cells in the fragments immediately after excision, and on the changes that occur in the sections during the cultural period. The observations designed to obtain these represent a separate phase of the investigation and they are accordingly presented separately in a section that immediately precedes the presentation of the absorption results.

MATERIALS AND METHODS

The material of this investigation is taken from maize roots and represents sections each 1·5 mm. in length taken successively from the tip to 9·0 mm. from it. Large numbers of corresponding sections are assembled. The collection of corresponding sections is subdivided into groups of 20, each group being cultured separately for periods up to 72 hours in a 25-ml. flask with 2 ml. of 0·01 N. KCl in water or of 0·01 N. KCl in a 2 per cent. solution of fructose. Samples of three flasks are taken at intervals from the series of cultures of the same section. The amount of potassium in each flask is determined and the lengths and breadths of all the sections in each group are measured. This is the procedure followed in the main series of experiments. Certain experiments were designed to investigate the effect on absorption of transferring the tissue from a solution containing only sugar to one containing sugar and potassium chloride. In this case after preliminary exposure to the sugar solution the tissue was removed from the experimental flask and without further treatment was transferred immediately to a second flask containing the sugar and potassium chloride solutions. In certain phases of the investigation, estimations of the potassium, protein, and sugar contents of the tissues have also been made.

General experimental techniques. The sections are taken from roots of a standard length that have been cultured in standard conditions. The parent roots are obtained from germinated seeds which have been incubated on moist ter-paper in Petri dishes in the dark for 48 hours. A standard volume of ater is added to each filter-paper before the seeds are placed on it. Only ots that are 2–2·5 cm. in length are used as parent material. These are parated from the seeds from which they have developed and transferred groups of ten to the cutting apparatus designed by Brown and Broadbent 950). Serial sections are obtained from ten roots simultaneously.

The cutting operation is repeated with successive groups until the requisite umber of sections has been obtained. The sections are distributed into roups of 20 and each group is placed in a 25-ml. flask with 2·0 ml. of the xperimental fluid. The flasks of a single experimental series are then transerred to the shaking apparatus used by Brown and Sutcliffe (1950), in which hey are shaken with a circular motion in a water-bath which is maintained t 25° C. The flasks are covered with a black paint to exclude light.

At intervals of 12 or 6 hours a sample of three flasks is taken from the group f flasks carrying corresponding sections. Each flask of each sample is treated eparately and each sampling occasion therefore yields three replicate values. he figures given below are each a mean of three such values.

When the sample has been taken the contents of each flask is poured on to sintered glass filter, the fluid from the culture being collected in a filter tube. The tissue on the filter is washed and the washings added to the filter tube, he contents of which are finally made up to 10 ml.

After washing, each group of sections is transferred to a microscope slide nd the lengths and breadths of each are measured microscopically against a alibrated micrometer eyepiece using a 2-in. objective. A mean length and a mean breadth are calculated from the twenty separate measurements. From these two mean values a mean volume is calculated. With the second and subsequent fragments in the series a single measurement for breadth is made at about the midpoint of the section, and volume is estimated on the assumption that the fragment is a simple cylinder. With the first fragment in the series the breadth measurement is made at the broadest point at the basal end. This fragment tapers to the apex and volume is calculated on the assumption that the shape is conical.

As indicated above, the design of the investigation requires that many of the determined values are reduced to a unit cell basis. The number of cells in each section is determined by the method of Brown and Rickless (1949) as modified by Brown and Broadbent (1950).

Figures are given for the number of non-vacuolated cells in each section. These are determined at the same time as the total number of cells and the counts are based on the standards for the non-vacuolated cell given by Brown (1951). It may be emphasized that since the counts are based partly on the apparent absence of a vacuole after treatment with chromic acid, and since the vacuole in the early stage of development is likely to collapse as the result of this treatment, with the section in which early vacuolation is occurring, the counts are likely to give an exaggerated estimate of the number of non-vacuolated cells.

Chemical methods. Potassium in the external fluid and in the tissue is determined by the technique of Hibberd and Stout (1933). The only modification introduced into the procedure recommended by these workers is that the precipitation with the sodium cobaltinitrite reagent is carried out in the silica crucible in which the initial ashing and acid treatment has been done. The determinations of the initial internal content of potassium in the tissues are based on samples of 100 sections.

Protein nitrogen is estimated on a group of 300 sections. These are ground and suspended in water. Total nitrogen is determined on an aliquot of this suspension and soluble nirogen on another after precipitation with trichloracetic acid. Protein nitrogen is estimated from the difference between these two sets of determinations. The total nitrogen in each fraction is determined by the micro-kjeldahl technique using the method given in Milton and Waters (1949).

In certain connexions the total sugar content of the tissue has been determined. For this purpose sections are extracted with hot 95 per cent. alcohol and, after evaporating the alcoholic solution under reduced pressure, the residue is redissolved in water. The sugar content of the aqueous solution is determined by the method of Hagedorn and Jensen (Strepkov, 1937) after hydrolysis of the sucrose with invertase.

General experimental design. The results for the main group of experiments given below involve 4 or 6 samples taken at intervals of 12 hours. All the results for any one series have not been obtained within a single experiment, but from two or three. Since a series of sections can be obtained from any given set of roots it is more convenient within a single experiment to obtain values for the same sampling occasion with different sections than for different sampling occasions with one section.

In certain series the cultures have been continued for 72 hours, in others for 48 hours. It has been found that excised tissues tend to become moribund if they are cultured for longer than 48 hours in the absence of a supply of sugar, and all the experiments in which fructose has not been supplied have therefore not been continued for longer than 48 hours. All tissues remain healthy up to 72 hours if sugar is supplied. The series involving the culture of the first fragment in the presence of sugar have, however, only been maintained for 48 hours. This is due to the fact that, in the later phases of the investigation, tips could not be obtained which developed consistently in culture and gave consistent results.

The growth and absorption of each section has been examined in the presence and absence of fructose. The growth of the tissues is frequently more vigorous in sucrose than it is in fructose. Fructose has been used, however, throughout the present series of experiments since growth with it is only about 10 per cent. less than it is in sucrose, and with it the contamination of the cultures with bacteria is less than with sucrose.

The effect of supplying calcium chloride has been investigated. It has been found that it has little effect on the absorption of potassium. In this respect e tissue of these experiments apparently differs from that used by Viets 944) and by Jacobsen, Overstreet, King, and Handley (1950). In the main ody of experiments the potassium chloride has been supplied without a ultivalent cation.

As indicated above, the present series of experiments involve 6 groups of ections, which are designated below as S_1, S_2, S_3, S_4, S_5, and S_6. The first ccupies the zone in the parent root 0–1·5 mm. from the tip, the second ·5–3·0 mm., the third 3·0–4·5 mm., the fourth 4·5–6·0 mm., the fifth 6·0–·5 mm., and the sixth 7·5–9·0 mm. Since the sections are of equal length e values for each section may be plotted against the serial order of the ections. This mode of presentation emphasizes the changes that occur along he course of the root, and some of the data have therefore been presented n this form.

GROWTH DATA

Growth data required in the interpretation of the absorption results are iven here. These are in two groups: (*a*) those showing the state of the average ell in each section immediately after excision, and (*b*) those showing the rowth of the tissues and therefore of the cells after excision.

State of cells after excision. The data obtained immediately after excision how the changes that occur in the cell as it develops from the meristematic o the fully expanded state in the root. The data presented show the changes hat occur in volume and protein content during this development. They are ased on measurements of the total number of cells in each section, on the umbers of non-vacuolated cells, and on the volumes and protein contents f the successive sections.

The total number of cells, the number of non-vacuolated cells, and the average volume of the cell in each section are shown in Table I.

TABLE I

Total numbers (thousands) of cells, N, numbers of non-vacuolated cells, NV, and volume per cell, VC (ml. × 10⁻⁸), in successive sections

	N	*NV*	*VC*
S_1 (0–1·5 mm.)	110	75	0·7
S_2 (1·5–3·0 mm.)	80	37	1·9
S_3 (3·0–4·5 mm.)	27	1·5	6·0
S_4 (4·5–6·0 mm.)	16	—	10·8
S_5 (6·0–7·5 mm.)	12	—	14·8
S_6 (7·5–9·0 mm.)	10	—	18·1

It is evident from the data of Table I that the minimum number of cells per unit length of root is reached at approximately 9·0 mm., which indicates that growth ceases at about this point. At the same time it may be noted that although there is only a slight decrease in the number of cells between the fifth and the sixth fragments the volume per cell nevertheless increases considerably. Clearly in this region the increase in volume is due mainly to an increase in breadth. A similar increase in breadth after length increase has ceased has already been observed in the bean root by Robinson and Brown (1952). The data of Table I show that from the meristematic to the fully expanded state there is a 20-fold increase in volume.

The number of non-vacuolated cells in each fragment is an important determination since it defines the limit of the meristematic zone and the transition to that of early vacuolation. The data show that about 70 per cent. of the cells in the first section are meristematic and that this section therefore covers the meristem in the parent root. The recorded data suggest that about 40 per cent. of the cells in the second section are non-vacuolated. For reasons that have been discussed in the previous part it is probable that this is an exaggerated estimate, and it may therefore be accepted that the second section covers the zone of early vacuolation. In the third and subsequent sections the number of non-vacuolated cells is negligible. Clearly the first section differs from all the others of the series in being composed almost entirely of meristematic non-vacuolated cells.

The protein nitrogen contents of the different sections and of the average cells in the different sections are shown in Table II.

TABLE II

Protein–N per section, NS (g. × 10⁻⁵), and per cell in each section, NC (g. × 10⁻¹⁰)

	NS	*NC*
S_1	1·00	0·91
S_2	1·02	1·27
S_3	0·43	1·61
S_4	0·33	2·09
S_5	0·20	1·66
S_6	0·20	2·03

Per section, protein increases from the first to the second section and decreases from the second to the sixth. Per cell, the protein content increases from the first to the fourth section and thereafter probably remains constant. It is significant that although the volume increases 20-fold, the protein–N per cell only doubles. In this connexion the form within which the cell expands in the root is of some importance. The cell does not expand uniformly but predominantly in the longitudinal direction. It grows as a cylinder, and the increase in surface area is therefore directly proportional to the increase in volume. Thus a 20-fold increase in volume involves approximately the same increase in surface area and the data indicate that, since there is only a twofold increase in protein during the course of growth, protein is reduced per unit surface to a tenth of the initial value.

The results given in Tables I and II are similar to others described by Brown and Broadbent (1950) and by Robinson and Brown (1952). They differ from the earlier series, however, in showing that although the greatest increase in protein content coincides with the phase where the greatest increase in length is occurring, nevertheless a slight increase in length may still ccur after increase in protein has ceased. This may be a differentiating aracteristic of the position in maize roots. On the other hand, the present ries of data are not as extensive as the earlier and definite conclusions regarding the relation between protein content and volume increase cannot be based n them.

Growth after excision. The growth of the different sections after excision is hown by the data of Table III.

The change in length and volume of the different sections in the presence nd absence of fructose is shown in Table III.

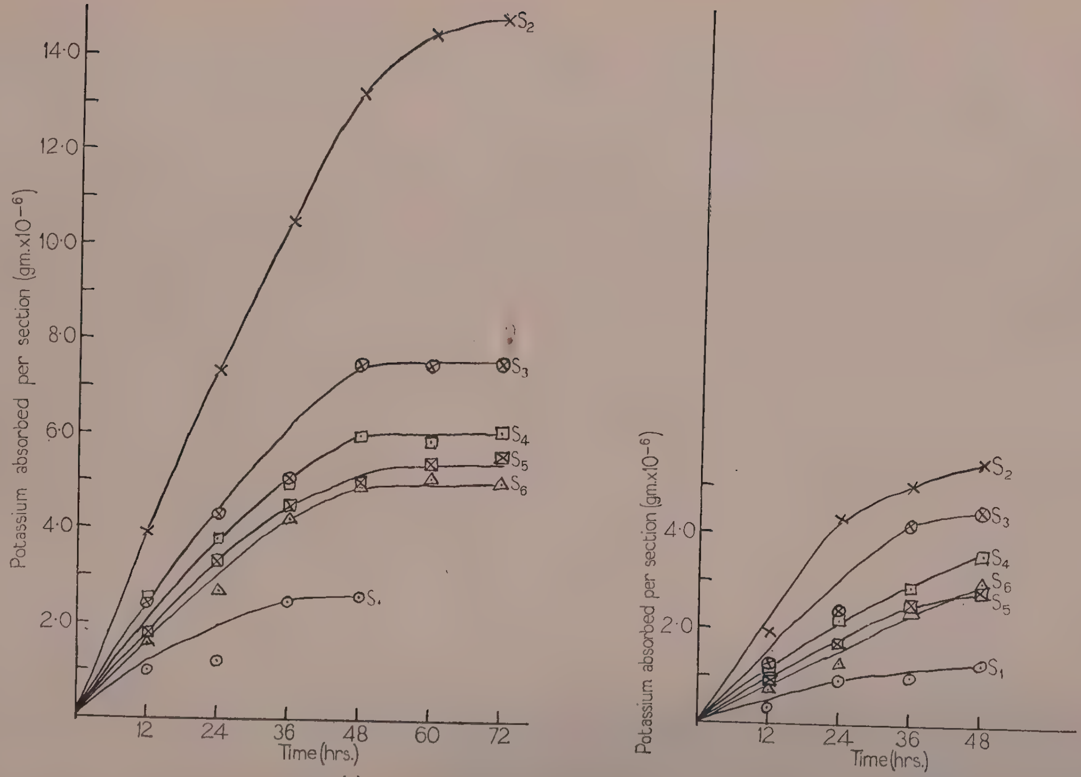
TABLE III

Lengths (mm.) and volumes (mm.^{3}) with (F+) and without fructose (F−); T time in hours

	T	S_1		S_2		S_3		S_4		S_5		S_6	
		F+	F−	F+	F−	F+	F−	F+	F−	F+	F−	F+	F−
Lengths	0	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5
	12	1·7	1·7	2·9	2·2	2·0	1·8	1·6	1·6	1·6	1·6	1·4	1·5
	24	2·3	2·2	3·9	2·7	2·0	1·9	1·7	1·6	1·7	1·7	1·7	1·7
	36	2·4	2·3	4·5	2·8	2·0	2·1	1·8	1·7	1·8	1·8	1·7	1·8
	48	2·7	2·4	4·9	2·9	1·8	2·0	1·8	1·8	1·8	1·8	1·8	1·8
	60	—	—	5·0	—	2·1	—	2·0	—	1·8	—	1·6	—
	72	—	—	5·0	—	2·2	—	2·0	—	1·8	—	1·7	—
Volumes	0	0·8	0·8	1·5	1·5	1·6	1·6	1·7	1·7	1·8	1·8	1·8	1·8
	12	0·9	0·8	3·3	2·3	2·3	2·0	2·0	1·9	1·9	1·9	1·9	1·9
	24	1·1	1·0	4·7	2·9	2·6	2·3	2·3	2·1	2·2	2·2	2·2	2·2
	36	1·5	1·1	6·2	3·4	2·8	2·5	2·4	2·2	2·4	2·4	2·3	2·4
	48	1·6	1·3	6·7	3·5	2·9	2·6	2·4	2·4	2·4	2·4	2·4	2·6
	60	—	—	6·9	—	2·8	—	2·5	—	2·4	—	2·2	—
	72	—	—	6·9	—	2·9	—	2·4	—	2·4	—	2·3	—

It is an important feature that during growth of all sections except the first, ell division does not occur, and therefore these changes in length or volume epresent corresponding changes in the constituent cells. In the first section limited number of cells may divide. It is clear, however, that whatever the xtent of division, if the whole fragment has not expanded considerably, as t has not in these experiments, then the individual cells have also not expanded. Division is a complicating feature only when the section increases considerably in length.

It is evident that in all series changes in length and volume occur during the experimental period. In the presence of fructose, with the first section, the volume doubles and growth probably continues for 48 hours; with the second it quadruples, and growth ceases at 48 hours; with the third it doubles and growth ceases at 36 hours; and with the fourth it increases about 40 per cent. and growth ceases at 24 hours. The increases with the fifth and sixth sections are similar to those of the fourth. In the absence of fructose growth is more limited and it tends to cease earlier than when fructose is present. Thus with the second section in the absence of fructose, the volume approximately doubles and the increase only continues for about 36 hours.



(*a*) (*b*)

FIG. 1. Increase in potassium absorption with time in different sections (*a*) in presence, (*b*) in absence of fructose. Figures attached to each curve indicate serial numbers of sections

The results of Table III are similar to an earlier series described by Brown and Sutcliffe (1950), who also found that the most vigorous growth in excised maize root tissues is given by the section which occupies the zone 1·5–3·0 mm. from the tip. The earlier series of results also showed that growth is more vigorous when sugar is supplied.

The expansion of the tissue after excision differs from the corresponding process in the intact root in one important respect. Whereas in the intact root expansion of the cell is accompanied by an increase in protein, in the isolated section it is not. In the course of growth of the isolated section in the presence of fructose the protein content does not change. As shown in Table II, the different sections carry different quantities of protein at the beginning of the experimental period. These quantities do not change significantly in the presence of fructose whatever the extent of growth. In the absence of fructose protein may decrease slightly, and this change undoubtedly limits the significance of the results obtained with this experimental treatment.

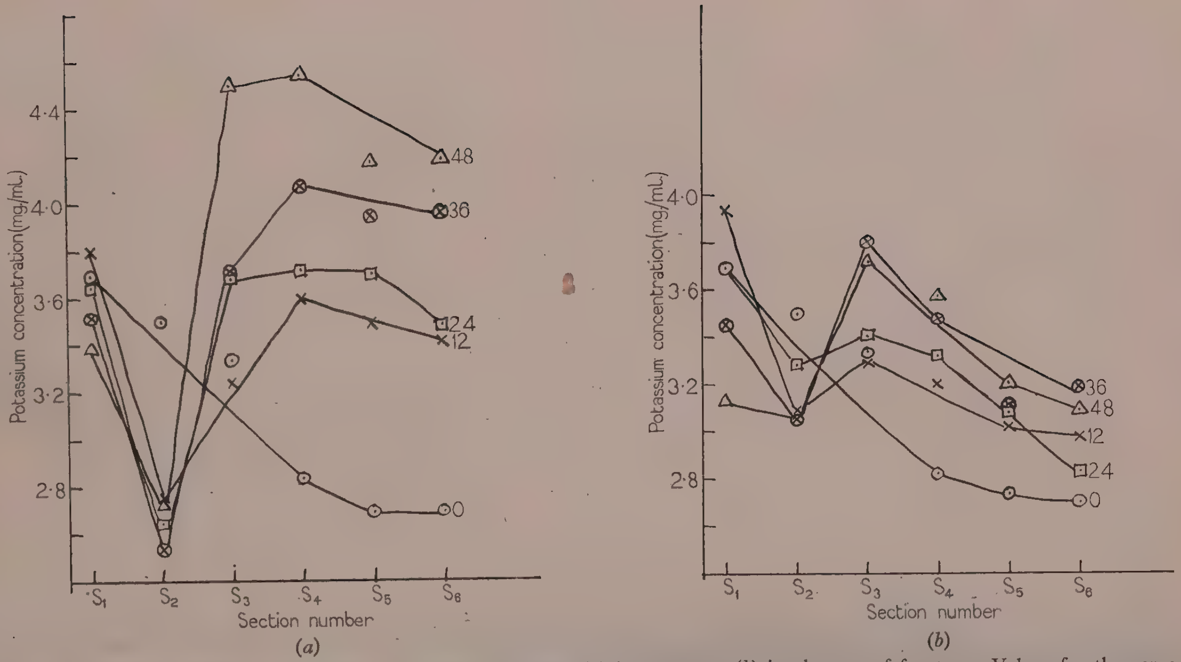
ABSORPTION OF POTASSIUM

Since the initial potassium content may determine later absorption, the results of the determinations of the potassium content of the several sections immediately after excision are considered first. Subsequently the following data for the different sections at different stages of the experimental period are given: (*a*) total absorption per section; (*b*) internal concentration of potassium; (*c*) absorption per unit protein; (*d*) absorption per unit cell; (*e*) potassium content per cell. In a final part certain data on absorption by the second section are given separately.

Initial potassium content. The initial potassium content per cell is given in Fig. 5 by the curves labelled *O*.

The calculated concentrations in successive sections are given by curve *O*, in Fig. 2. It is evident that while initial content per cell increases steadily from the first to the sixth section, concentration is highest in the first and decreases steadily from this to the sixth. Penston (1931) working with root tissues has also found that the concentration of potassium is greater in the meristem than it is elsewhere.

Absorption per section. The increase in absorption with time in the presence and absence of fructose in the different sections is shown in Fig. 1*a* and *b*. The rates of absorption are, of course, given by the slopes of the several curves. All the curves are more or less linear during the first 36 hours, indicating that during this period the rate of absorption is more or less constant. It is evident that both in the presence and absence of fructose the rate is lowest in the first fragment, highest in the second, and decreases progressively from the second to the sixth. In the presence of fructose absorption in all series ceases at about 48 hours, although in the second section it may continue until about 60 hours. In the absence of fructose absorption continues throughout the experimental period, but since the rate between 36 and 48 hours is considerably lower than it is in the previous 12-hour period, it is probable that absorption ceases at



(*a*) (*b*)

FIG. 2. Internal potassium concentration in different sections (*a*) in presence, (*b*) in absence of fructose. Values for the same sampling occasion are plotted against the serial order of the sections. Figures attached to curves indicate sampling time in hours.

4 hours. Thus concentration data based on the values for 48 hours may be taken as representing the steady state. The effect of supplying fructose is considerable. Absorption is always substantially greater in the presence than it is in the absence of fructose.

Finally, it may be noted that a comparison between the data of Tables III and Fig. 1*a* and *b* shows that absorption always continues after growth has ceased.

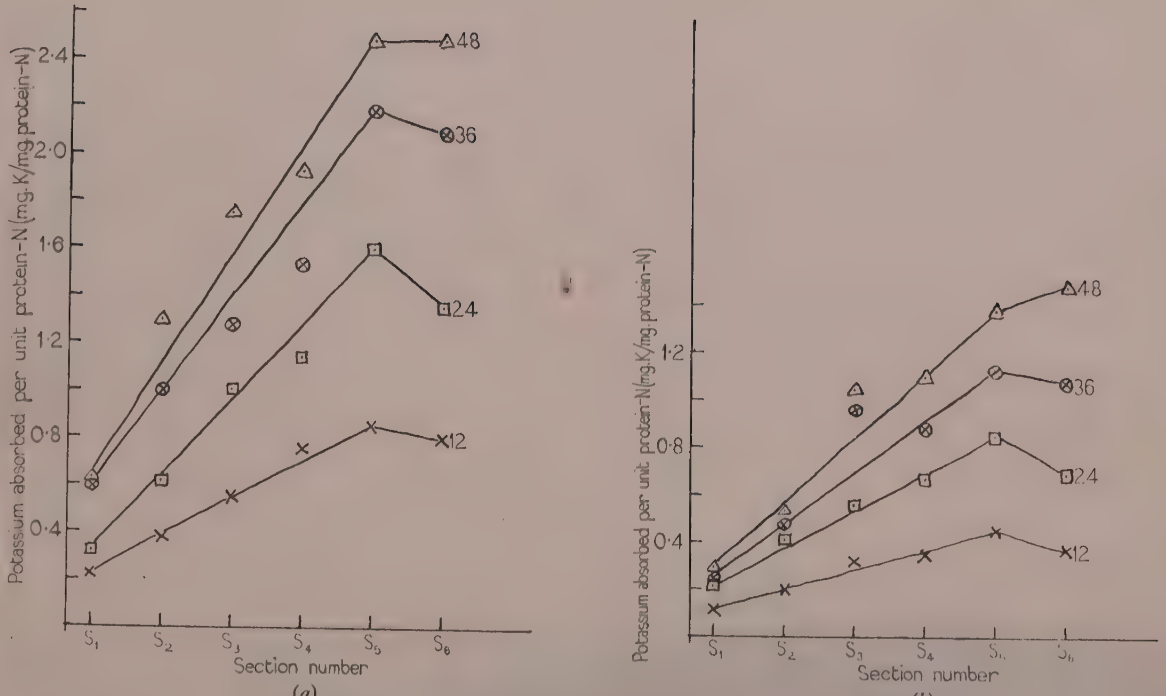
Change in internal concentration. Concentration values for each section and for each sampling occasion have been calculated from the volume and total content data. The total content is the sum of the initial content and the quantity absorbed. The concentration values are given in Fig. 2*a* and *b*, and they show that the internal concentrations at corresponding times differ considerably. It is convenient to consider the results obtained with and without fructose separately.

With fructose the concentration in the first section, although it does not hange greatly, tends to decrease with time. In the second section, it decreases harply during the first 12 hours, less steeply from 12 to 36 hours, and then creases gradually until the steady state is reached at 60 hours (the data for his section are plotted against time in Fig. 7). With the second section the nal concentration is lower than the initial, and it is lower than the corresponding value for the first and considerably lower than the corresponding alue for the third section. With the third and subsequent sections the concentration increases with time; the values at corresponding times tend to be ore or less the same, although there is some indication that at 48 hours, when he equilibrium concentration has been reached, it tends to decrease from the hird to the sixth section.

In the absence of fructose the position is different. With the first and second ections the concentrations change with time in approximately the same way s they do with fructose. Supplying fructose, however, does not apparently ncrease the final concentration in these two sections. In the first the concentrations are approximately the same. In the second they are probably slightly ower in the presence than they are in the absence of fructose. With the third nd subsequent sections concentrations again tend to increase with time, but he final concentrations are considerably lower than they are with fructose. There is little difference between the values for the second and third sections. With the third and subsequent sections the final concentration tends to decrease along the series and it does so much more sharply than when fructose s supplied.

It may be noticed that the differences between the several series in the bsence of fructose cannot be attributed to corresponding differences in the nitial internal content of sugar. This has been determined, and it has been found that it is approximately the same in all sections from the second to the sixth.

Many of the changes and differences in internal concentration are the result of corresponding differences in growth. With sections 3 to 6 in the presence



(a) (b)

Fig. 3. Absorption per unit protein N. Scheme of presentation as in Fig. 2.

nd absence of fructose, the internal concentration increases with time. In ese sections growth is relatively small. With section 2 growth is extensive, nd although absorption is rapid the internal concentration decreases during e first 36 hours. Growth involves primarily an absorption of water and the ata indicate that water is absorbed relatively more rapidly than potassium nd as a result the internal concentration decreases. Similarly with section 1 oncentration tends to decrease with time and again water absorption is more apid than potassium absorption.

Absorption per unit protein. Values per unit protein have been calculated nd these are shown in Fig. 3*a* and *b*. It may be pointed out that these alues are based on total absorption after different times and the increasing evel does not therefore represent a rate per unit protein increasing with time. t is evident that the rate increases progressively from the first to the sixth ragment, and that it is always lower when fructose is not supplied.

Absorption per cell. These data have been converted to a unit cell basis and n this form they are given in Fig. 4*a* and *b*. In Fig. 4*a* the values for 60 nd 72 hours are not given since they are approximately the same as those for 8 hours. The differences in the rates of absorption calculated on a per cell nd a per section basis are striking. Whereas the rate on a per section basis ncreases from the first to the second and then decreases from the second to he sixth, the rate per cell increases steadily from the first to the sixth. Clearly he high rate in the second is due primarily to the relatively large number f cells it contains. The rate increases from the first to the second section since he effect of the increase per cell is relatively greater than the reduction in he number of cells. The rate per section decreases from the second to the ixth since the effect of a decreasing number of cells is relatively greater than hat of an increasing rate per cell.

Potassium content per cell. Data for the total potassium content per cell are iven in Fig. 5*a* and *b*. The total content is the sum of the initial content nd the amount absorbed. The data show that throughout the experimental eriod the total potassium content increases from the first to the sixth section. t may be emphasized that the curves for 60 to 72 hours are very close to hat for 48 hours, indicating that when the maximum content per cell has een reached, this also increases from the first to the sixth fragment. Finally, t is significant that the absolute change in content per cell is least in the first ragment and increases progressively to the sixth.

Absorption by second section. It is clear from the data presented above that the second section is in many respects exceptional. Two features are considered further here: (1) although during the first 36 hours there is at least a threefold increase in surface area, nevertheless the rate of absorption remains constant; (2) the internal concentration is lower at the end than it is at the beginning of the experimental period.

The rate of absorption remains constant while the internal concentration is decreasing; this suggests that the inflow is independent of surface area, and that the rate remains constant since in the early stages of growth the outflow

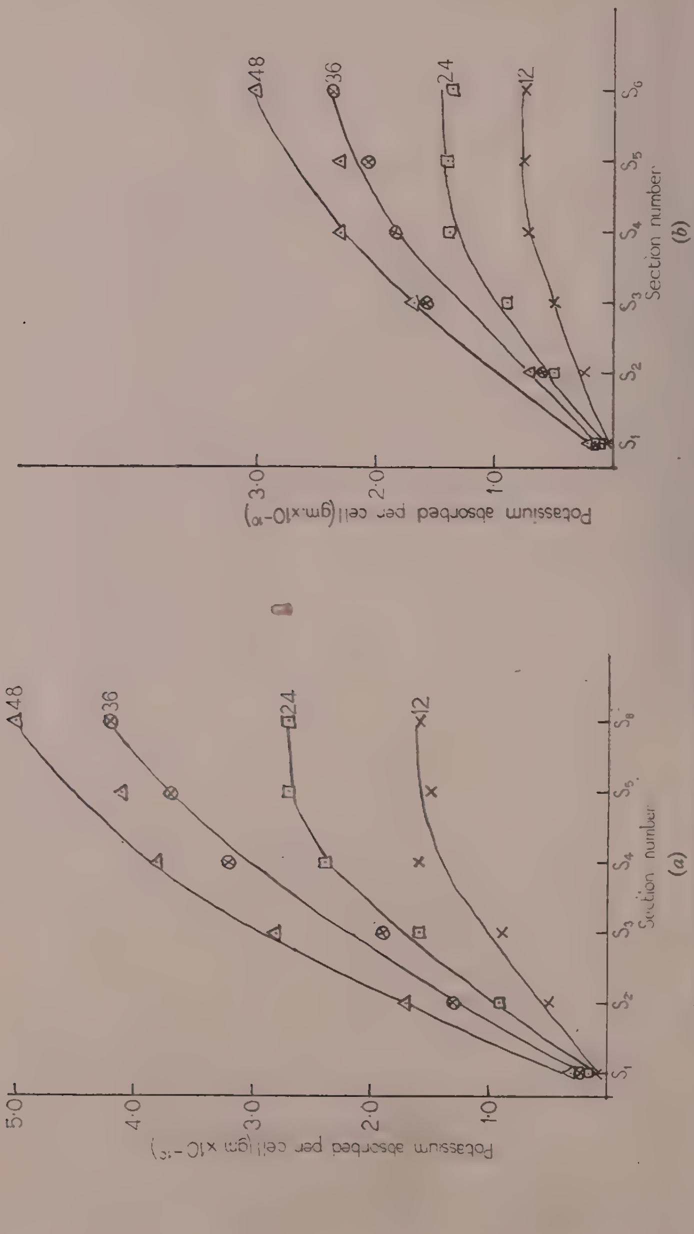


FIG. 4. Absorption per unit cell in different sections. Scheme of presentation as in Fig. 2.

small relative to the inflow. This possibility may be examined by deter-ining the rate of absorption after various periods of growth in a potassium ee medium. Experiments incorporating this situation have been undertaken. roups of second sections have been cultured in sugar for 12 and 24 hours nd then transferred to a medium containing sugar and potassium and the sorption measured in the normal way. The results are shown in Fig. 6. he effect of culturing sections on a potassium free medium must be to duce the internal concentration of potassium since during the period in hich potassium is not supplied growth occurs and water is absorbed. It has een shown that the internal concentration may also be reduced by a slight aching out of potassium. The changes in the internal concentration that ccur when the tissue is transferred from a sugar to a sugar and potassium edium at 24 hours are shown in Fig. 7. Now if the approximately constant ate of absorption when potassium is supplied at the beginning of the experi-ental period is due to a constant rate of inflow, then the same rate of absorp-ion should be given when the internal concentration is reduced. The data of ig. 6 show that when the tissue is transferred at 12 hours, although the nternal concentration has been reduced to at least half the original value, evertheless the rate of absorption is the same as it is when potassium is upplied at the beginning of the experimental period. An accelerated absorp-ion is only given after 24 hours in a potassium-free medium when the internal oncentration has been reduced to less than a third of the original value. Further, the acceleration after 24 hours, although it occurs, is not large. Clearly the data suggest strongly that the constant rate of absorption in the main series of experiments with the second section is due to a constant rate of inflow which does not increase with increase in surface area.

After 36 hours the rate of absorption tends to decrease with time and finally ceases at about 60 hours. It is significant, however, that absorption ceases before the internal concentration reaches the original level after the tissue is excised. Since the rate of inflow probably does not change during the course of the experimental period, the low final concentration suggests that the outflow increases. If the outflow relative to the inflow does not change, then the internal concentration should return at least to the original state. The outflow may increase as a result either of a change in permeability or of an increase in surface area. Both changes may be involved, but the assumption that increase in surface area is an important element in the situation is at least consistent with the results of other observations of this investigation. In particular, if permeability is a decisive factor, then the relative effect of outflow might be expected to be greater in the absence than in the presence of sugar. Evidently it is not since the final concentration with the second section in the absence of sugar may be higher than in its presence although the surface area is smaller and the protein content may be slightly lower. The conclusion that outflow depends on surface area is fully consistent with the assumption that it represents a diffusion process.

It may be noted that the results obtained with the second section show that

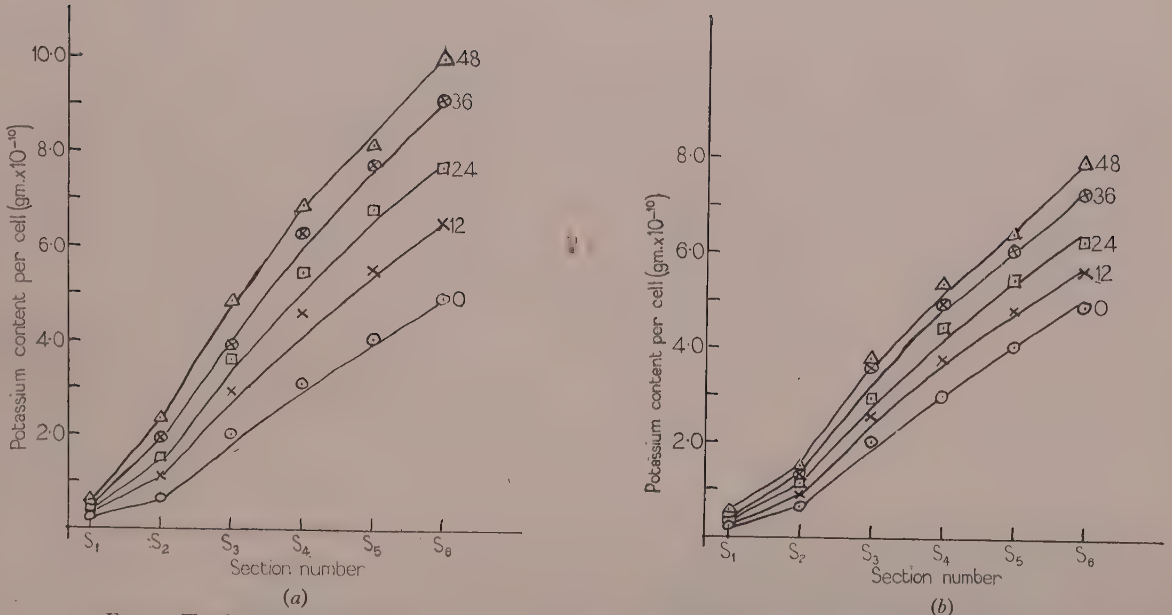


FIG. 5. Total potassium content per unit cell in different sections. Scheme of presentation as in Fig. 2.

asorption is not controlled by growth. If it were, then a clear correlation tween the two processes might be expected. In fact the data show that asorption continues after growth has ceased, and it continues for different nes according to the treatment. When sugar is supplied it continues for hours and when it is not supplied for at least 24 hours after growth has ased. Growth is significant for the absorption process only to the extent at it affects surface area—at least with excised tissue.

At the same time it is clear from the data of Figs. 6 and 7 that permeability anges may be more readily induced when the cell is growing than when it is ature. The steady-state concentrations when potassium is supplied at the ginning of the experiment and after growth has begun are not the same. has been found that this difference is not developed with mature tissue such that of the sixth section. In the second section when potassium is supplied 4 hours after growth has begun the final length is approximately the same it is when it is given throughout the experimental period. The different nal concentrations cannot therefore be attributed to different surface areas. he differences suggest that during growth the cytoplasm is in a particularly bile state. In this state potassium (or chloride ions) can react with it in such way that its permeability decreases to potassium itself. When the labile state as been traversed potassium chloride can no longer have any effect. Thus hen the salt has been supplied during only half the growth period its effect n permeability is less extensive than when it has been supplied throughout. he conclusion that the reaction of the cytoplasm to potassium or chloride ons changes during growth is consistent with the suggestion of Robinson nd Brown (1952) that the metabolic pattern changes as the cell traverses ne growth phase.

DISCUSSION

The absorption data assembled above are considered in relation to (*a*) the ffect of sugar; (*b*) the differences in rate and final concentration given by the uccessive sections; (*c*) the differences in rate and absolute contents given by he cells in the successive sections; and (*d*) absorption in the intact root. It nay be emphasized here that the experimental situation carries an important mplication for the interpretation of these data. The rate data for the first 12 hours are obtained partly before extensive changes have occurred in the sections and they therefore provide some indication of the position in the ntact root. Also since the successive sections contain cells in progressively advanced stages of development, they show how the changes in cell growth that proceed in the root affect absorption. The data for the period after 12 hours show how absorption is affected by cell expansion which occurs in different circumstances independently of the parent root.

Effect of sugar. It is clear that absorption in the tissues of these experiments incorporates a phase which is closely linked to metabolic processes. This is emphasized by the ratios of the internal and external concentrations for the stage when absorption ceases at 48 hours. Those ratios for the successive

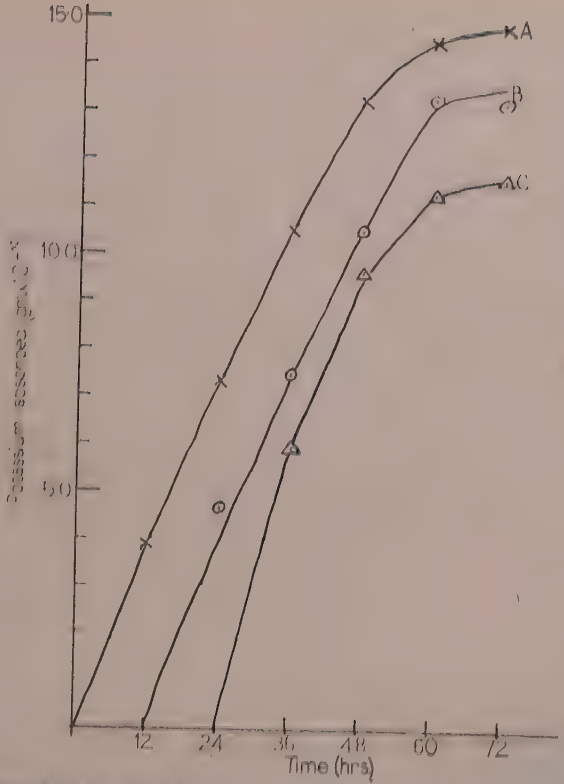


FIG. 6. Absorption by second section in presence of fructose: *A*, when potassium is supplied at the beginning of the experimental period; *B*, when supplied after 12 hours; *C*, when

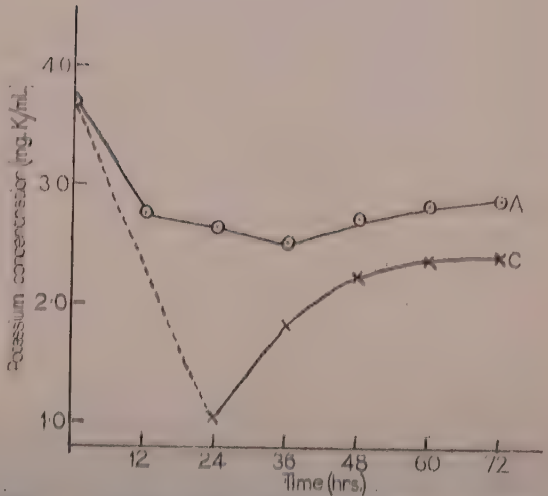


FIG. 7. Internal concentration in second section in presence of fructose when supplied *A*, at beginning, and *C*, 24 hours after beginning of experimental period.

ragments when fructose is supplied are as follows: S_1, 1:9·3; S_2, 1:10·7; S_3, 1:14·1; S_4, 1:13·6; S_5, 1:12·1; S_6, 1:12·1.

The importance of metabolic activity is further emphasized by the results of supplying sugar. When sugar is supplied the rate of absorption of potassium s always increased. Roberts, Roberts, and Cowie (1949) have recently shown that in *Escherichia coli* the absorption of potassium is stimulated by the provision of sugar, and they attribute this effect to the formation from the sugar of a specific carrier for potassium. A similar mechanism may be involved in this case. On the other hand, the present series of results are more readily interpreted in terms of a generalized metabolic stimulation to the tissue. Hoagland and Broyer (1936), Prevot and Steward (1936), and Humphries (1950) described a similar effect from the provision of sugar to roots, and they interpreted their results in the sense proposed here. Brown and Sutcliffe (1950) have shown that when the second sections (from the apex) are immersed in a sugar solution, the sugar is accumulated in the tissue, and there is a considerable increase in the rate of respiration. In general terms it may be concluded that the provision of sugar maintains metabolic activity at a level at which energy is available for the promotion of potassium absorption.

It is significant that, whereas sugar always increases the rate of absorption, the effect of it on final concentration varies with the extent to which the tissue grows. When growth is limited or does not occur (as in sections 5 and 6) the provision of sugar leads to a relatively high concentration when absorption ceases. When growth is vigorous, on the other hand, the final internal concentration of potassium in the presence of sugar may be lower than it is in its absence. The increase in final concentration when sugar is supplied and growth does not occur is no doubt a consequence of an enhanced inflow without a corresponding effect on outflow promoted by a change in permeability or surface area. With a higher rate of inflow a corresponding rate of outflow can only be maintained with a steeper concentration gradient and the steady state concentration is consequently high. When growth occurs the provision of sugar enhances the expansion of the tissue. When sugar is supplied not only is the inflow enhanced but the outflow is also increased, at least partly, as a result of a greater surface. Although there is a greater inflow the proportion that escapes is increased and a steady state may be established with a relatively low final internal concentration.

The changes in internal concentration and rate of absorption in the second section, together with the effects of sugar discussed above, suggest that inflow is clearly dependent on metabolic states and is independent of surface area. Outflow, on the contrary, while varying with surface area is not directly affected by metabolic conditions. It may be suggested that inflow depends on a number of discrete inflow units dispersed throughout the cytoplasm. As the cell grows in an isolated fragment the number of these inflow units does not change and they simply become more widely dispersed. Since the number of units does not increase, inflow is not accelerated as surface area expands. The activity of the units, however, is closely dependent on metabolic states. As the availability of metabolic energy increases so also does the activity of each inflow unit and hence the accelerating effect of sugar on inflow. Outflow in contrast to inflow is closely dependent on surface area and is accelerated by sugar only to the extent that surface area is increased. When sugar is provided absorption is always stimulated. The final concentration, however, when expansion has ceased will depend on the relative effects on inflow unit activity and on expansion. If the latter is relatively the greater, then final concentration may be depressed as a result of a relatively high surface area dependent outflow.

It may be emphasized that this interpretation is based on isolated fragments, in which protein does not increase during growth.

Differences between sections. One of the most prominent features of the whole array of data given above is the extremely low absorption by the first section, which is composed of predominantly meristematic cells. The low absorption may be determined to a certain extent by a relatively high initial internal concentration of potassium. At the same time it may be noted that the internal concentration at corresponding times in the first section is similar to that in the second in being considerably lower than it is in other sections of the series. In the second the low concentration is a consequence of the extensive growth that occurs in it. This, however, is not the position with the first. In this connexion the first may be compared with the third section. In both of these, although the degree of expansion in culture is about the same, the rates of absorption and the internal concentrations when sugar is supplied are different. The differences, it may be suggested, are due to different structural conditions in the constituent cells. They are certainly not due to different metabolic activities, since per fragment (and it is the fragment differences that are at present in question) the rate of respiration is higher in the meristem than it is elsewhere (Brown and Broadbent, 1950). While the cells of the first fragment, however, are predominantly non-vacuolated, those of the third all carry large prominent central vacuoles. At the same time it is clear that the higher rate of absorption and the higher concentration developed in the third fragment cannot be attributed to the larger volume of water incorporated in the vacuoles. These conditions may, on the other hand, represent effects promoted by structural entities associated with the vacuole. Of these the most prominent is the tonoplast, the membrane which separates the vacuole from the cytoplasm, and the data therefore suggest the possibility that the capacity to accumulate solutes and therefore to promote absorption is at least due partly to the properties of this membrane. The low degree of accumulation in the first section may be due to the small proportion of cells in this region that are vacuolated.

Since the sections are all 1·5 mm. in length, the differences in rates during the first 12 hours indicate corresponding differences in absorption per unit area of tissue and of cell surface. The data indicate that per unit area absorption is lowest in the first section, highest in the second, and decreases progressively from this to the sixth. The factors that determine the low rate in the st have been discussed above. The decrease from the second to the sixth volves conditions other than the development of the vacuole. It is certainly ot due to initial differences in the internal concentration of potassium, since is is highest in the second and lowest in the sixth. On the other hand, it may e due to a rate of inflow which is highest in the second and decreases from is to the sixth. In terms of the interpretation proposed above this would dicate that the number of inflow units per unit area decreases from the cond to the sixth section.

It is significant that a gradient in final concentrations corresponding to the radient in the initial rates of absorption per unit area is not always observed. he second section which has a higher initial rate than the third develops considerably lower final concentration. The origin of this difference has een considered above. When growth does not occur a variation in final ternal concentration corresponding to the variation in rate per unit area is ound when sugar is not supplied. From the third to the sixth section when ugar is not supplied there is a downward gradient. There is also some indication of this gradient within this group of sections when sugar is provided. Vith sugar, however, some growth occurs in the third section, and this tends o reduce the final concentration and thus to reduce the gradient.

Absorption per cell. The successive fragments of this series incorporate cells n progressively advanced stages of development. The average cells in the uccessive fragments represent the development from the meristematic to the ully vacuolated state which involves an increase in volume with a consequent nlargement in the mass of the vacuole, and an extension of surface area.

When the absorption data for the successive fragments are reduced to unit cell basis they show that the rate of absorption increases. The rate data how the effect of growth on absorption when this growth occurs in the intact oot. In terms of the hypothetical scheme proposed in this paper this would uggest, since the initial absorption is not determined by internal concentration, that per cell the number of inflow units increases as the cell enlarges.

It has been shown above that when growth occurs outside the root the rate of absorption does not change with the expansion of the cell. Cell growth in the excised fragment, however, does not involve an increase in protein. Clearly since increase in surface area and vacuolar volume as such do not promote a higher rate of absorption and presumably of inflow in the excised fragment, the increase in rate of absorption with growth in the root cannot be attributed to these changes either. The data suggest that the increase in the rate must be attributed primarily to increase in protein or to some property associated with it. It is probable that the increase in protein represents an increase in the mass of cytoplasm with a consequent increase in the number of inflow units.

The section data discussed above indicate that per unit area of cell surface the rate of absorption decreases as the cell expands in the root. In an earlier part of this paper it has been shown that during normal growth although protein increases the quantity per unit surface decreases. This feature of the growth of the system, it may be suggested, is the origin of the decrease in rate of absorption per unit area.

The position is complicated by the fact that absorption per unit protein increases as the cell expands. Since surface area and vacuolar volume as such have little effect on the rate, the increase per unit protein must be attributed either to an acceleration of the activity of the inflow units or to a differentiation of the cytoplasm in such a way that the proportion of these per unit volume increases. Both possibilities may be involved since it has been shown (Kopp, 1944; Brown and Broadbent, 1950) that respiration per unit protein increases as the cell expands and that the growth of the cell may involve changes in the activities of different enzymes per unit protein (Robinson and Brown, 1952).

The absolute content of potassium increases as the cell enlarges both in the root and after excision. In the initial stages after excision the content must depend on vacuolar volume and rate of absorption, and in the final stages on volume and steady state concentration. The effect of rate of absorption and final concentration are seen in the difference of absolute content in the presence and absence of sugar, and the effect of vacuolar volume in the change in the content at corresponding times as the cell expands both in the root and after excision.

The relatively small change in content with time in the meristematic cell is, of course, a consequence of a small volume, and conversely the large change in the fully expanded cell is an expression of its much greater volume.

Absorption in the intact root. Prevot and Steward (1936) and Steward, Prevot, and Harrison (1942) have shown that, in barley roots which have been cut into fragments of 1·0 or 0·5 cm., absorption of both anions and cations is highest in the apical fragment and decreases progressively from the tip to the base of the root. The present series of results amplify the earlier in showing that even within the terminal centimetre there are likely to be considerable variations in the rate of absorption per unit length. These different groups of results are clearly of some significance in relation to absorption by the root as a whole. With regard to the growing zone the present series of results suggest that absorption is likely to be slow in the meristem, rapid in the region of early vacuolation, decreasing, per unit length, from this point to the limit of the zone. Such is likely to be the position when the observations are made over short intervals of time. The present series of data provide no indication as to the probable succession in rates when the observations are prolonged and the position is complicated by growth changes and transfer of solutes into the xylem. The factors that determine variation in absorption rates are, of course, likely to be the same in isolated fragments and in intact roots over short periods. A high rate of absorption immediately behind the meristematic zone in the intact root is likely to be due as much to numbers of cells involved as to peculiarities of the metabolic state.

Finally it may be noted that, although when the root is sectioned the rate of absorption is lowest in the meristem, nevertheless in the intact root the highest concentration of potassium is found in this zone. The difference is robably due to the mechanism of delivery of potassium to the meristem and the subsequent expansion of the cells. It is possible that potassium is supied to the apex along the procambial strands which may reach to within or 3 mm. of the apex. The cells that are charged with potassium from this urce expand, and as they do so water is probably absorbed more rapidly ian potassium and as a result concentration decreases and decreases proressively from the apex to the limit of the growing zone.

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The Stimulation of Growth by Low Temperature in Embryos of *Heracleum sphondylium* L.

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SUMMARY

Low temperature of 2–5° C. accelerates the rate of formation of amino acids from reserve proteins in the endosperm of *Heracleum sphondylium*. It also brings about the accumulation of glycine and arginine in abundance. Experimental culture of excised embryos on synthetic media shows that these acids are particularly beneficial to embryo growth whilst alanine, the predominating acid at room temperature, is not. It is suggested that the rapid growth following low-temperature treatment results from the action of low temperature on protein breakdown.

INTRODUCTION

In a previous paper (Stokes, 1953) it has been shown that a low temperature of 2–5° C. is necessary to bring about the breakdown of the protein reserves in the endosperm of *Heracleum sphondylium*. Otherwise the embryo is starved of nitrogenous compounds necessary for its growth within the seed before germination can take place.

At a low temperature of 2–5° C. embryos grow exponentially within the seed and germinate after 9–12 weeks. At room temperature, however, the growth of the embryo is very much slower and ceases entirely after 6 weeks, when the size reached is only half that at germination (Stokes, 1952*a*).

THE EFFECT OF TEMPERATURE ON THE AMINO-ACID CONTENT OF THE ENDOSPERM

In resting seeds one-third of the total nitrogen is in soluble form. At room temperature this remains constant, but at 2° C. the proportion is rapidly increased. Furthermore, at 15° C. the embryo utilizes little of the soluble nitrogen available, whereas at 2° C. it rapidly synthesizes protein. This suggests that the composition of the soluble nitrogen fraction is different at the two temperatures and is in a form suitable for the embryos to take up only at low temperature. The soluble amino-acid fractions present in the seeds have therefore been analysed by the paper chromatography technique.

Method. Moist seeds were kept for 8 weeks in (i) a constant temperature of 20° C. and (ii) in a refrigerator at a temperature fluctuating from 2° C. to 5° C.

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he embryos were then removed and the endosperms were killed in ether, ried at 100° C. for 1–2 hours, and ground to a fine powder.

Resting seeds were soaked overnight at room temperature to facilitate the xcision of the embryos. The endosperms from these were treated in the same ay.

The amino-acids were extracted from the powdered endosperms by aking in 70 per cent. ethanol for 24 hours (1 ml./10 mg. endosperm material sed). The extraction was repeated.

The two extracts were then combined, evaporated to dryness *in vacuo*, and e residue was finally dissolved in a small volume of distilled water (2 μl. of ater for each 1 mg. of endosperm material extracted).

Total amino nitrogen in the extracts was estimated using the colorimetric inhydrin method of Moore and Stein (1948); 25 μl. of each extract were sed for a determination, any ammonia present being removed by the method f Fowden (1951) before the addition of the colour reagent.

The amino-acids present in the extracts were determined by two-dimenional paper chromatography (Consden, Gordon, and Martin, 1944). 100 μl. f the final solution were used for each chromatogram. Water-saturated henol in the presence of ammonia was used as the first developing solvent, nd the *n*-butanol-acetic acid mixture of Partridge (1948) as the second. After he removal of the second solvent the chromatograms were sprayed with a ·1 per cent. solution of ninhydrin in water-saturated *n*-butanol. The aminocids were located as blue-purple spots after heating the chromatograms for –10 minutes at 100° C.

One-way chromatograms were also run in each of the above two solvents.

In interpreting the results of chromatographic analysis it is necessary to ear in mind the inherent limitations of the method.

All other soluble materials in the extract interfere to a greater or lesser xtent in amino-acid chromatography, tending to produce serious 'streaking' f the amino-acid spots if too much extract is applied to a chromatogram. The maximum quantity of extract which can be used for a chromatogram is herefore limited to that which gives good separation of spots for the more bundant acids.

This may well mean that acids present in much lower concentrations are oo dilute to be detected in the amount of extract used. Failure to obtain a eaction for any particular amino-acid does not therefore necessarily prove its bsence from the extract.

Histidine, for example, is a usual constituent of plant extracts, yet owing to its relatively low concentration and weak ninhydrin reaction under the conditions of colour development used for paper chromatograms, it is frequently not identified, unless some more specific spraying agent is used. Minor constituents become too dispersed to recognize in two-way chromatograms. From the analyses recorded in Table II it will be noticed that the presence of proline and α-aminobutyric acid was confirmed only in the one-way chromatograms run in butanol.

RESULTS

Quantitative estimations of total amino-nitrogen contents of endosperms as given in Table I show that amino-acids accumulate in the endosperms at both temperatures, and it is seen that the rate of accumulation is greater at low temperature than it is at room temperature. The rate of utilization by the embryos is also much greater at 2° C. than at 20° C. (Stokes, 1953).

TABLE I

Total α-amino-nitrogen content of endosperms

Resting seeds	0·36 µg. amino nitrogen/mg. dry wt.
After 8 weeks at 2° C.	0·53 µg. amino nitrogen/mg. dry wt.
After 8 weeks at 20° C.	0·44 µg. amino nitrogen/mg. dry wt.

Results of chromatographic analysis, given in Table II, show that during development at 2° C. there was a very marked increase in the arginine content of the endosperms. This was accompanied by a decrease in the lysine and or ornithine (which are indistinguishable by this method) and after 8 weeks they were not detected. There was also a marked loss of alanine.

Similar changes in these acids took place during development at 20° C., although to a much less degree, and these were accompanied by a decrease in glycine content.

Thus after 8 weeks' development the endosperms at 2° C. were rich in arginine and poor in alanine and the other basic amino-acids, whilst endosperms at 20° C. had retained a larger proportion of alanine but were comparatively poor in glycine and arginine. The relative quantities of the other amino-acids present was much the same at both temperatures.

These results are in general agreement with previous reports that the accumulation of soluble nitrogenous compounds in the endosperm is more rapid at low temperature than at room temperature (Stokes, 1953; Vallance, 1952). It is also shown, however, that it is the balance of the diverse amino-acids rather than their number which is affected by temperature.

The majority of amino-acids are present in equal quantities at both temperatures, and yet there is very little soluble nitrogen taken up by the embryo at room temperature. It may therefore be assumed that those amino-acids which enable embryos to grow during after-ripening are those which are more readily available at low temperature than at room temperature. The analyses performed after 8 weeks' development show that there is more glycine and arginine present in endosperms at 2° C. than at 20° C., and this suggests that these particular acids may be necessary for embryo growth. Alternatively these may be accumulation products, and the comparatively low alanine content at 2° C. may indicate that this acid is being rapidly absorbed by the embryo.

It is therefore difficult to assess the individual roles of glycine, arginine, and alanine from these analytical data alone, but the problem is further discussed in a later section. There can, however, be no doubt that these amino-acids exert a profound influence on embryo development.

TABLE II

Chromatographic analysis of amino-acids in endosperms of Heracleum sphondylium

he relative concentrations of constituents may be gauged by the intensity of colour and the size of spots, and is indicated by the number of crosses)

	Resting seeds	After 8 weeks at 2° C. (1 week before germination)	After 8 weeks at 20° C. (embryos stopped growing)	
ystine	+	++	++	Considerable increase at both temperatures
ysine	++	—	+	Marked decrease, especially at 2° C.
rnithine				
istidine	—	—	—	No reaction obtained
rginine	+	+++	++	Marked increase, especially at 2° C.
spartic acid	+++	+++	+++	
lycine	++	++	+	Decrease at 20° C.
erine	++	+	+	Decrease at both temperatures
lutamic acid	+++	+++	+++	
hreonine	+	—	—	
-Alanine	+++	+	++	Marked decrease, especially at 2° C.
roline	+	+	+	Identified when run in butanol only
-Alanine	+++	+	++	Marked decrease, especially at 2° C.
-aminobutyric acid	+	+	+	Identified when run in butanol only
-aminobutyric acid	++	++	++	
yrosine	++	+	+	
ethionine	++	++	++	
aline				
henylalanine	—	—	—	No reaction obtained
so-leucine	++	++	++	
eucine				
itrulline or glutamine	+	+	+	

THE CULTURE OF EXCISED EMBRYOS ON SYNTHETIC MEDIA

In an attempt to obtain further information on this matter a technique was levised for studying the growth of excised embryos on synthetic media in rder to determine the effect of various carbohydrates on embryo growth, and o determine the possible effects of individual amino-acids on embryo levelopment.

Method. Embryos were grown in boiling-tubes on filter-paper slope standing in a liquid medium as described more fully in another paper (Stokes 1952*b*).

Seeds were removed from the pericarps and sterilized for 30 minutes i 1 per cent. calcium hypochlorite solution according to Wilson (1915) an White (1934). The embryos were then excised under sterile conditions an placed in previously sterilized culture tubes. Only rarely was there an infection following this treatment, and sterilized seeds showed no ill-effects o germination.

The basic medium (medium 1) used was that described by Tukey (1938 It was made up of 10 g. KCl, 2 g. KNO_3, 2·5 g. each of $CaSO_4$, MgSO $Ca_3(PO_4)_2$, $Fe_3(PO_4)_2$. To 1·5 g. of this mixture were added 20 g. of anala glucose and 1 litre of distilled water. The pH was approximately 6. Thi medium was then modified by substituting a variety of other sugars fo glucose, or by replacing the KNO_3 with an amino-acid, equimolecular wit respect to available nitrogen.

Unless otherwise stated 20 embryos were used for each treatment. The were grown at 20° C. in artificial light supplied by fluorescent tubes givin an intensity of 50 foot-candles.

RESULTS OF PRELIMINARY EXPERIMENTS

Growth of embryos was studied when they were supplied with: (1) distille water only; (2) medium I (mineral solution with 2 per cent. glucose); (3 mineral solution only, at 2° C., 20° C., and 30° C. Sets of whole seeds wer similarly set up.

1. Embryos grown on water showed only a slight enlargement, but ther was no development and the swelling was probably caused by water uptak only.

2. Very rapid growth was obtained on the solutions in which minerals an glucose were supplied both at 20° C. and 30° C. and healthy seedlings wer produced. Growth at 30° C. was more rapid at the beginning, but the subsequent development was erratic and distortions of the seedlings indicated tha 30° C. was a less satisfactory temperature for culturing than 20° C. At 2° C growth was very slow. A temperature of 20° C. was therefore used for al subsequent cultures.

3. There was a little growth of embryos on mineral solution only, som even doubling their size, but development was slow and never reached beyon this point.

Embryos are therefore entirely dependent upon an external supply o sugars for their development.

There was no germination of whole seeds during 10 weeks at 20° C. o 30° C., but at low temperature germination was normal and not affected b the presence of glucose or minerals in the medium.

THE EFFECT OF DIFFERENT CARBOHYDRATES ON GROWTH OF EMBRYOS

Sets of embryos and whole seeds were grown on the following media: edium 1, mineral solution + 2 per cent. glucose (Analar); medium 2, neral solution + 2 per cent. fructose (Kerfoots biochemical reagent); edium 3, mineral solution + 2 per cent. sucrose (Analar); medium 4, neral solution + 2 per cent. galactose (B.D.H.); medium 5, mineral lution + 2 per cent. mannose (Analar); medium 6, mineral solution + er cent. starch (Analar). Controls were also set up on mineral solution and tilled water.

Good growth of excised embryos was obtained on all media containing gars, and no distinction could be observed between the effects of glucose, ctose, galactose, and mannose. Growth of excised embryos on sucrose was ttle more rapid in the early stages, but there was no difference in the final ges of development. Embryos were apparently unable to utilize starch and haved as on mineral solution only. There was no germination of whole ds during 8 weeks.

As has been noted, there was no germination of whole seeds on glucose edium at 20° C. or 30° C. The most likely reason for this is that sugars fail penetrate the endosperm which is many cells thick and covered with a er of suberin.

To test this the endosperm was scraped away until only a thin layer covered e embryo on the flat side of the seed. Ten such scraped seeds were placed t side down on the filter-paper in tubes containing each of the different edia Nos. 1–6, mineral solution only, and distilled water only. Whole seeds re used as controls on all the media.

The scraped seeds went brown around the wounded area and several were fected during the scraping process. All the embryos were therefore excised d examined after 8 weeks as the contamination was becoming serious.

TABLE III

Pseudo-germination of scraped seeds on different media at 20° C.

Weeks	Minerals + dextrose	Minerals + fructose	Minerals + mannose	Minerals + galactose	Minerals + sucrose	Minerals + starch	Mineral solution only	Water
4	Severe contamination	—	1	1	—	—	—	—
6		1	2	—	—	—	—	—
7		—	3	—	—	—	—	—
8		—	—	—	2	—	—	—

umbers of seeds germinating from samples of 10 are given cumulatively in able III. There was no germination in the scraped seeds on mineral solution d water alone. The embryos from these were examined and found to be in e same stage of development as those in whole seeds. The process of raping and the resultant increase of oxygen tension therefore had no effect the growth of the embryo.

Embryos from scraped seeds on sugar media were also examined ar measured. It was found that they were quite normal in so far as the stage development and length measurement were concerned, but in all of them the: was an abnormal swelling of the portion of the embryo immediately below tl area of scraped endosperm tissue. In some only the radicle was affected, bı in others where the scraped area was larger the cotyledons were also swolle although it must be emphasized that the stage of development and lengt were not advanced beyond normal for seeds at room temperature.

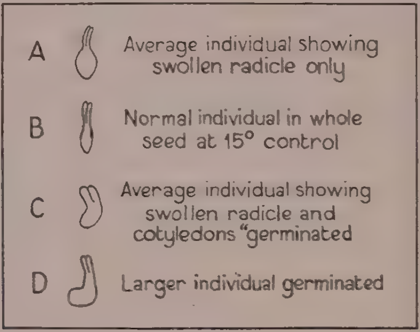
A
Average individual showing swollen radicle only
B
Normal individual in whole seed at 15° control
C
Average individual showing swollen radicle and cotyledons "germinated
D
Larger individual germinated

Fig. 1. Typical embryos developed in scraped seeds on media containing sugars. For explanation see text.

Fig. 1 shows the appearance of the swollen embryos. *C* and *D* are ind viduals which were recorded as germinated. It can be seen that none of tl rapid extension growth associated with germination had taken place, and tl degree of swelling was much the same in both 'germinated' and ungerminate embryos. The reason for apparent germination is that in a few seeds the thi layer of endosperm gave way under the pressure of the abnormal swelling an the whole embryo was slightly extruded by the force of its own expansio Since this is not true germination it explains why the radicle showed r further elongation.

The availability of additional sugars therefore results only in a localize swelling and does not increase the rate of development or growth of tl embryos in the seed. This confirms a previous suggestion that it is neither tl composition nor the quantity of carbohydrates available which limits embry growth within the seed at room temperature.

THE EFFECT OF VARIOUS FORMS OF NITROGEN ON THE GROWTH OI EXCISED EMBRYOS IN CULTURE.

Embryos were grown as before on mineral solution plus 2 per cent. glucos but the KNO_3 was replaced by nitrogen in the following forms: medium glycine 0·1150 g./l.; medium 8, *dl*-alanine 0·2728 g. l.; medium 9, *l*-glutam acid 0·2513 g./l.; medium 10, *dl*-serine 0·3217 g. l.; medium 11, arginir 0·1593 g./l.; medium 12, alanine+serine+arginine. Embryos were al wn on medium 1 containing KNO_3 as the source of nitrogen. The nitrogen ntents of media 7, 9, and 11 equalled that of medium 1. Media 8 and 10 ntained twice this quantity.

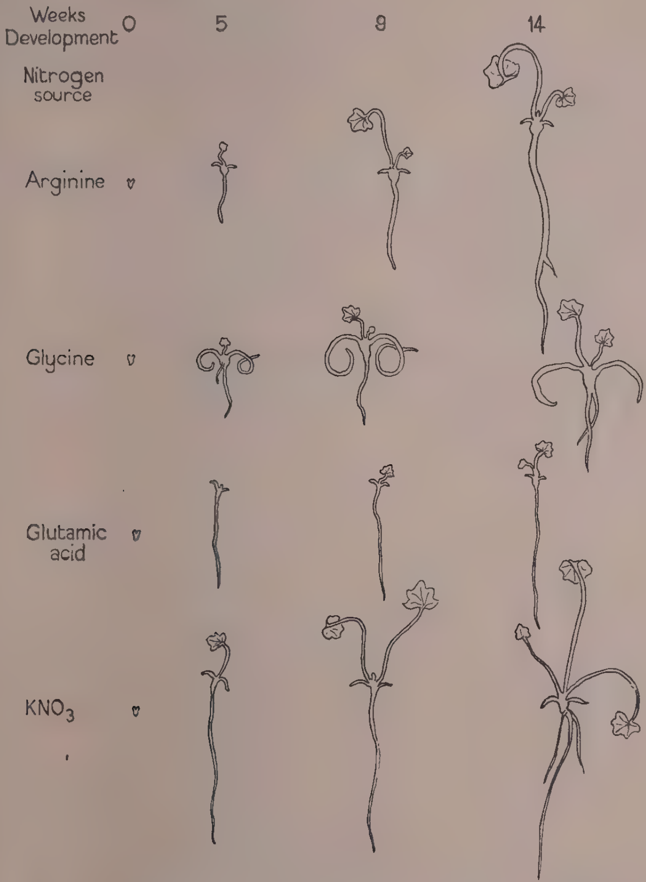

FIG. 2. The effect of the form in which nitrogen is supplied on the morphology of embryos grown in culture.

Fig. 2 shows typical examples of embryos at various stages of development on KNO_3, arginine, glycine, and glutamic acid. There was no growth on serine or the control solution and none on alanine after the first fortnight.

Of these, the cultures with KNO_3 most resemble seedlings produced from after-ripened seed in size and general appearance. Seedlings supplied with arginine are also similar to those from the seed, but the stem is bulbous in appearance and the roots are noticeably thickened. Glycine produced small stocky seedlings with well-developed cotyledons but a general appearance of distortion. Embryos grow on glutamic acid, but there is very little cotyledon or leaf development, the bulk of the seedlings consisting of a long root. Thos grown on the amino-acid mixture did not grow so well as when supplied wit arginine alone.

The results of these experiments agree with those of Virtanen and Linkol (1946) in which plants fed with different amino-acids showed variations i morphology. Nightingale (1937) also found that of a large number of substances tried nitrate was the most suitable form in which to supply nitrogen t higher plants in culture.

The successful growth of seedlings obtained with glycine and arginine a the sole sources of nitrogen shows that these amino-acids which are produce in the endosperm at low temperature are beneficial to embryo growth. Th poor development of seedlings supplied with alanine alone and with othe amino-acids suggests that the low alanine content of endosperms at lo temperature results from its slow rate of formation rather than from its rapi uptake by the embryo.

It is thus seen that low temperature not only increases the rate of hydrolysi of the endosperm protein reserves, but it also brings about the formation o those particular amino-acids which are beneficial to embryo growth and whic are not formed in such abundance at room temperature.

A COMPARISON OF THE EFFECT OF TEMPERATURE AND NUTRITION ON THE RATE OF EMBRYO GROWTH IN CULTURE AND IN THE SEED

It was not possible to make dry-weight determinations of cultured embryo since the labour involved prevented the use of sufficiently large numbers, bu from length measurements it was possible to make a rough assessment o growth rate. During the early stages of development accurate length measurements were made with an eyepiece micrometer, but after the plumular bu had begun to open the growth was distributed amongst the various leaves an secondary roots so that measurements were less accurate.

In Fig. 3 the rates of growth and development of embryos in culture ar compared with those of embryos growing 'naturally', that is to say growing i the seed for 9 weeks at low temperature, germinating, and developing int a seedling in the warmth at 20° C. (*ACBE*). *AD* shows growth in the seed a room temperature.

The growth rate of embryos cultured at 20° C. (*ABE*) is high and, apart from a week or two while the plumular bud develops, it is fairly constant.

In the seed, however, as has been shown (Stokes, 1953), the rate of embryo growth is limited by the supply of nutrients, and since the rate o hydrolysis of endosperm proteins is higher at low temperature, the rate o growth in the seed is higher at low temperature (*AC*) than at room temperature (*AD*). None the less the very low temperature does have a retarding effect and embryos do grow much more rapidly at room temperature when supplied with suitable nutrients in culture (*ABE*). The growth of embryos in culture at low temperature is shown with crosses and it coincides with growth in the seed at low temperature (*AC*).

The three curves shown may therefore be described thus: AD = growth starved embryos at room temperature; AC = growth of well-nourished nbryos at low temperature (in the seed or in culture); AB = growth of well-ourished embryos at room temperature (in culture).

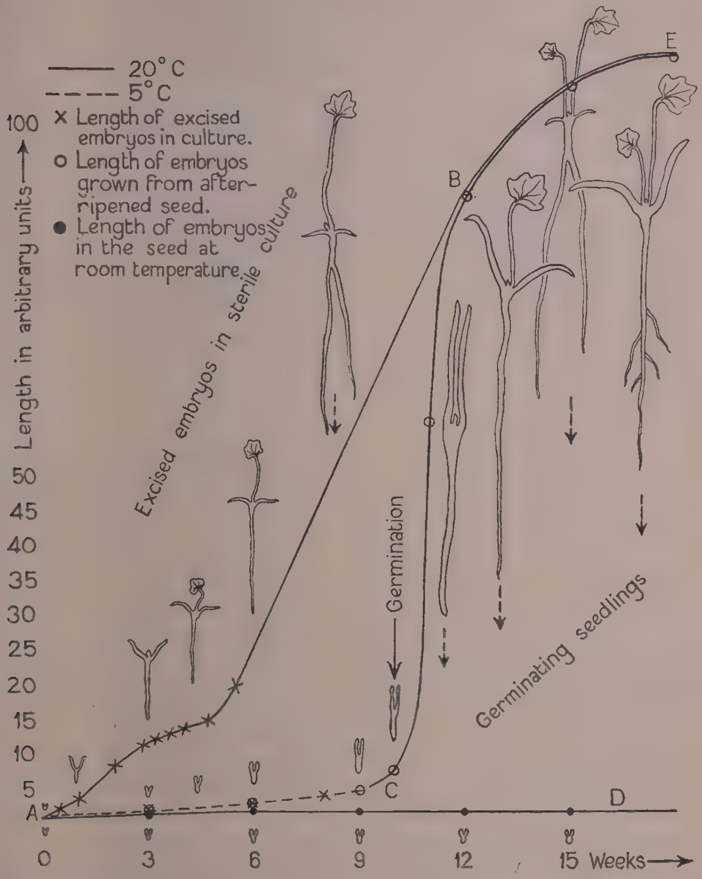

FIG. 3. The rate of growth of embryos in the seed at low temperature (AC), in the seed at room temperature (AD), and in culture at room temperature (ABE) and at low temperature (AC). CBE shows the rate of growth of seeds germinating at room temperature. The drawings show the stages of development relative to size at any given time under the different conditions.

Embryos in the seed at low temperature grew comparatively slowly in the early stages, but after 9 weeks when nutrition was no longer limiting they were transferred to 20° C. The rate of growth during germination was then very rapid for about 3 weeks (CB). The result was that after 15 weeks there was little difference in appearance or size between embryos grown in culture at room temperature and embryos grown from seed at low temperature, and both followed the growth curve *BE*.

The differences observed in rate of growth between embryos grown in culture and embryos grown in the seed at low temperature (*ACB* and *AB*), large as they are, however, are not so striking as the very great differences in rate of development especially during the first few weeks.

Excised embryos growing in culture grow quickly in size and develop quickly so that after 4½ weeks there is a little seedling 15 times the original size with the first plumular leaf unfolding. By comparison the embryo in the seed germinates and reaches 75 times its original size before the first leaf starts to unfold. In the seed, therefore, the rate of growth is slower and the final size reached before plumular emergence is much larger.

Growth and development are, however, very rapid during germination, which compensates for the slow progress within the seed. This then produces, as has been shown, a seedling very similar to that produced by steady growth of embryos in culture. They would in fact be indistinguishable but for the fact that in the seed the prolonged enlargement phase before the development of the plumular bud results in the growth of much larger cotyledons than is the case with embryos grown in culture.

THE EFFECT OF LIGHT ON GROWTH AND DEVELOPMENT

The factor responsible for this difference of rate in early development may well be light. Embryos growing in the seed and in culture in the refrigerator (*AC*) were in the dark, whereas those grown in culture at room temperature were in the light (*AB*).

Embryos were therefore grown in culture at room temperature in both light and dark in order to determine the effect of light on development. Those in the light were in the conditions used for all other cultures, namely continuous illumination of approximately 50 foot-candles by fluorescent lamps. Those in the dark were in boiling-tubes covered completely with black paint and plugged at the top.

From the preliminary tests carried out it was found that the presence of light in the early stages accelerates the rate of development in comparison with those in the dark, which grew to a much larger size before the spreading of the cotyledons and emergence of the plumule.

The differences in rates of development between embryos cultured in the light and embryos growing in the seed may be partially accounted for by differences in illumination, and the rapid increase in the rate of development which occurs at germination is therefore probably effected by the presence of light at this time.

DISCUSSION

It is now shown that low temperature increases the rate of accumulation of amino-nitrogen compounds from the endosperm proteins of *Heracleum*. Not only this, but it also brings about the accumulation of those particular mino-acids most beneficial to embryo growth, and which are not found in uantity at higher temperatures.

The significance of this action is seen in the specific effects of individual mino-acids on embryo growth in culture. Both arginine and glycine give good mbryo growth in culture, and these are the amino-acids which accumulate n greater abundance at low temperature than at room temperature. Alanine, n the other hand, is relatively more abundant at room temperature, and it is ncapable of supporting embryo growth in culture.

Furthermore, when provided with a favourable source of nitrogen the omposition of the sugars available does not influence embryo growth, so that ossibility of any change in composition of endosperm sugars brought about y low temperature is immaterial to embryo development. The total amount f sugars available in the endosperm is more than sufficient to supply the rbohydrate needs of the embryo, so that the nutrients which are limiting to owth in the seed appear to be entirely nitrogenous.

There is therefore seen to be a very intimate connexion between embryo owth and the amount of protein synthesis possible in the embryo.

The formation of dwarf seedlings from non-after-ripened embryos Flemion, 1933, 1934; Flemion and Waterbury, 1945) is comparable with he growth of *Heracleum* embryos in the seed at room temperature. The only utrients which forced embryos can obtain from their cotyledons are comarable to those formed in the endosperm of *Heracleum* at room temperature, nd the stunted growth suggests that they also may be suffering from starvaon of nitrogen in a suitable form.

The action of low temperature in hydrolysing starch to sugar is often held o be responsible for the stimulating effect of low temperature on the growth f a number of plant organs. Many seeds require low-temperature treatment efore they will germinate, but it is characteristic of these that, like *Heracleum* eeds, they do not contain starch, whereas they do contain abundant storage rotein. Similarly, since the importance of protein synthesis in cell enlargement has been established (Brumfield, 1942; Brown and Broadbent, 1951), t becomes at least as probable that nitrogenous transformations are associated with the rapid extension growth of tree-buds, flower-buds, &c., which follows ow-temperature treatment so frequently. In organs which do not contain tarch, and in those which do as well, the widespread action of low temperature n stimulating extension growth may well be found to result from the hydroysis of proteins, as has been demonstrated in seeds of *Heracleum sphondylium*.

ACKNOWLEDGEMENT

I am greatly indebted to Dr. L. Fowden of this department who performed the analyses in Section 1.

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Studies in the Principles of Phytotoxicity

V. THE EFFECTS OF THE DEGREE OF NITRATION ON THE TOXICITY OF PHENOL AND OTHER SUBSTITUTED BENZENES

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SUMMARY

1. The relative toxicity of phenol, *o*- and *p*-nitro, 2:4-dinitro-, and 2:4:6-trinitrophenol was measured by up to five techniques. Determinations were made of the concentrations required to reduce by half (i) the growth rate of *Trichoderma viride* at pH 3, (ii) the frond multiplication rate of *Lemna minor* at pH 5·1–5·4, (iii) the respiration rate of yeast at pH 3, (iv) the respiration rate of infiltrated leaf disks of *Brassica alba* at pH 3, and (v) the number of *B. alba* seedlings surviving after spraying with buffered solutions of the compounds. Whenever possible toxicity was measured at a pH level below pK so as to eliminate the effect of pH on toxicity, but in some of the measurements with 2:4-dinitrophenol ($\mathrm{pK} = 4{\cdot}0$) and with picric acid ($\mathrm{pK} = 0{\cdot}8$) this was not feasible. It was then necessary to measure toxicity at a pH at which the compound was much dissociated; from the measured values of toxicity of dinitrophenol the toxicity at a lower pH was estimated from the generalized pH-activity relationship of Simon and Beevers (1952).

2. The relative toxicity of the nitrophenols is very similar whichever test procedure is employed. The first two nitrations are accompanied by large increases in toxicity, but with the third successive nitration there is a decrease.

3. The toxicity of the corresponding mono, di, and trinitro derivatives of anisole, phenetole, toluene, and nitrobenzene was determined using *T. viride*. In each series the first and third stages of nitration brought about large increases in toxicity but the intermediate one had little effect.

4. All the *p*-nitro compounds were more toxic than their *o*- isomers.

5. On the basis of the concepts put forward by Ferguson (1939) and Albert (1951) it is concluded that the toxic action of the nitroanisoles and nitrotoluenes is ‘structurally non-specific’, i.e. the effect is not dependent on the presence or absence of a particular chemical group in the molecule. With both the nitrobenzene and the phenol series the results suggest that the exact chemical structure is an important factor.

INTRODUCTION

ALTHOUGH it was known at the beginning of this century that nitrophenols were toxic to some insects, Tattersfield, Gimingham, and Morris (1925) were the first to carry out a comprehensive survey of the potential value of nitrophenols and related compounds for the control of insects or insect eggs. Subsequently these studies of relative toxicity were extended by other workers with a view to gaining some understanding of the mode of action of

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nitrophenols and of the factors which make one compound more toxic than another.

It has commonly been reported in the literature that toxicity increases in the series phenol, mononitrophenol, dinitrophenol, but that with picric acid, the product of a third nitration, there is a fall in toxicity. These changes in toxicity hold not only for insects but for other organisms such as bacteria and fungi, and the conclusion can be reached that these compounds are toxic to almost all types of organism.

In spite of the widespread use of 3:5-dinitro-*o*-cresol and 2:4:dinitro-6-*sec*-butylphenol as selective herbicides—see recent reviews by Norman *et al.* (1950) and Blackman *et al.* (1951)—only brief reports by Chabrolin (1943) and Crafts (1945) are concerned with the toxicity of other nitrophenols to angiosperms. The object therefore of the present investigation has been to assess the relative toxicity of a range of nitrophenols to higher plants, and at the same time to measure for comparative purposes their toxicities to other organisms such as yeast and the mould *Trichoderma viride*.

There has been comparatively little systematic work on the effect of the degree of nitration on the toxicity of simple benzene derivatives other than phenol and in consequence several other series of compounds have been studied. The parent compounds for these series were anisole (methoxybenzene), phenetole (ethoxybenzene), toluene, and nitrobenzene, and whenever possible each series included the *ortho* and *para* mononitro, the 2:4-dinitro, and the 2:4:6-trinitro derivative.

EXPERIMENTAL METHODS

In the first paper of this series (Blackman, 1952) it was emphasized that the most precise estimates of relative toxicity can be obtained by determining the amount of each compound required to bring about a standard response and that on statistical grounds it is desirable to select as standard a 50 per cent. reduction in the effect which is being measured. In the present investigation five different procedures have been employed for assessing toxicity, and since detailed descriptions have already been published only an outline of each test has been given together with the appropriate references.

Inhibition of the growth of Trichoderma viride (Simon and Blackman, 1949). The fungus *Trichoderma viride* was grown on buffered agar media containing a range of concentrations of the toxic substance, and the diameter of the mycelium on each plate was measured at suitable intervals. From these measurements the rates of radial spread were determined and the concentration required to halve the growth rate was interpolated from a graph of the growth rate against the logarithm of the concentration.

Inhibition of the respiration of yeast (Simon and Beevers, 1952). The rate of oxygen uptake of baker's yeast suspended in buffered glucose solutions containing the desired concentration of toxic material was measured in the conventional way with a Warburg manometric apparatus and the concentration required to halve the respiration rate was then determined graphically.

Inhibition of the growth of Lemna minor (Simon, Roberts, and Blackman, 1952). Plants of *Lemna minor* were grown under conditions of constant light intensity and temperature, floating on water-culture media containing graded concentrations of the toxic substance. The number of fronds was counted daily and the rate of increase of the logarithm of frond number taken as a measure of the growth rate at each concentration. The concentration of each substance required to halve the growth rate was determined graphically.

Inhibition of the respiration of vacuum-infiltrated leaf disks of Brassica alba (Simon *et al.*, 1952). The rate of oxygen uptake by 8·5 mm. disks, cut from leaves of *Brassica alba* seedlings, was measured with a Warburg apparatus. A buffered solution of the toxic substance was vacuum-infiltrated into the intercellular air-spaces of the disks so that the individual cells of the leaf tissue were bathed in the medium. As in the yeast experiments, toxicity was measured as the concentration required to halve the respiration rate.

Mortality of seedlings of Brassica alba (Simon *et al.*, 1952). The toxic materials were sprayed in buffer solutions on to mustard seedlings, grown in pots, by means of an apparatus in which the rate of travel over the pots and the output from the nozzle were so controlled that the rate of delivery was constant and equivalent to 100 gallons per acre. The number of plants sprayed with each treatment was counted before the experiment and the percentage mortality was determined from the proportion of plants surviving after a week. The concentration required to bring about a 50 per cent. mortality was found graphically.

Materials. The compounds employed were of laboratory grade and, apart from the samples of picric acid which are discussed later, no further purification was undertaken. As most of the compounds are sparingly soluble in water, the solids were first made up as stock suspensions (5–15 per cent.) by grinding in a ball-mill while the liquids were emulsified with a high-speed stirrer; in each case a small quantity of gelatinized alumina, a biologically inert wetting agent, was added. Dilutions were made from these suspensions and emulsions when they were required.

EXPERIMENTAL RESULTS

The relative toxicity of phenol and nitrophenols

The assessment of the relative toxicity of phenol and the nitrophenols presents a problem of general interest in the estimation of relative toxicity because these compounds are weak acids. It has already been established that changes in pH may greatly influence the toxic action of a weak acid in the pH range above the pK of the compound (Simon and Blackman, 1949; Simon and Beevers, 1952). Thus the toxicity of 2:4-dinitrophenol (pK = 4·0) decreases as the pH is raised above 4, but that of *o*-nitrophenol (pK = 7·2) remains constant up to pH 7. Hence the relative toxicity of these two compounds depends on the pH at which the observations are made. The effect of pH can, however, be eliminated by selecting a pH at which there is little ionization, i.e. pH levels below pK.

So far as possible this procedure has been followed in the present experiments. Since phenol and *o*- and *p*-nitrophenols have high pK values (10·0, 7·3, and 7·2 respectively), there was no difficulty in determining the toxicity at pH values below pK. However, in the case of 2:4-dinitrophenol (pK = 4) it was not always possible to carry out experiments at a sufficiently low pH, and in consequence it was necessary to estimate the toxicity at a low pH level from measurements made at a higher pH. Simon and Beevers (1952) have shown that, apart from a few clearly defined exceptions, for acids with a pK between about 3 and 9 the relationship between pH and activity is the same whatever the acid used or the test organism employed, and they found that this relationship could be expressed in the form of a curve which covered a very wide range of pH and fitted a large number of experimental observations. This curve has been used for estimating the toxic effect to be expected at a lower level of pH.

The concentrations of phenol and the mono- and dinitrophenols required to produce the standard response from each of the test organisms are shown in Table I, together with the pH levels at which the observations were made. Where the toxicity was measured at a pH greater than pK, the actual concentration is given as well as the estimate for a lower pH. For *T. viride*, yeast, and leaf disks of *B. alba*, the toxicity of 2:4-dinitrophenol was determined at about pH 3 and the estimated value of toxicity at lower pH levels is close to the measured value. However, the determination with *L. minor* was made at pH 5·4, the lowest pH at which satisfactory growth was obtained in Clark's solution. It can be seen that the estimated value is sixteen times less than the measured value.

In the previous paper of this series (Simon *et al.*, 1952) it was pointed out that when a weak acid, such as 3:5-dinitro-*o*-cresol, is sprayed on to a shoot the pH factor does not operate in the same way as in experiments like those with yeast and *T. viride* in which the organism is in direct contact with a relatively large volume of test solution. Thus, under the conditions of spraying the effect of pH on the toxicity of a weak acid is masked as a result of the relatively large bulk of seedling compared with the spray deposit and the greater buffering power of the plant tissues. The pH at which the toxic substances act upon the cells is therefore governed by the pH of the leaf tissues rather than by that of the spray droplets. The spray solutions in the present experiments were in fact buffered at pH 4, but the exact pH at which the materials acted is unknown. As a working hypothesis, the appropriate pH is presumed to be 6, and this value has been used to correct the experimental value for dinitrophenol.

In order to bring out the effect of increasing nitration on toxicity the mean concentrations required to produce the standard response in the several tests have been plotted against the degree of nitration (see Fig. 1). The concentrations given are those required at a pH below pK, whether measured directly or estimated from the experimental observations. It is clear that with each of the test organisms there is a very close agreement for the effects of single and

TABLE I

The molar concentrations of phenol and of three nitrophenols required to give the standard responses in each of the five tests. The pH at which toxicity was measured is given in parentheses after each figure. The concentrations in heavy type are estimates of the concentrations that would be needed at a pH below pK; the basis of this estimate is discussed in the text. pK values are taken from International Critical Tables (1939)

	50% reduction of growth rate of *Trichoderma viride*	50% reduction of respiration rate of yeast	50% reduction of growth rate of *Lemna minor*	50% reduction of respiration rate of infiltrated leaf disks of *Brassica alba*	50% mortality of seedlings of *Brassica alba*
Phenol pK = 10·0	$3{\cdot}1\times10^{-3}$ (3·7) $4{\cdot}1\times10^{-3}$ (5·4) $4{\cdot}6\times10^{-3}$ (6·5) $4{\cdot}0\times10^{-3}$ (8·0)	$3{\cdot}8\times10^{-2}$ (4·6)	$5{\cdot}1\times10^{-3}$ (5·4)	$1{\cdot}1\times10^{-2}$ (3·0)	$4{\cdot}8\times10^{-1}$
o-nitrophenol pK = 7·3	$8{\cdot}7\times10^{-4}$ (3·7) $8{\cdot}7\times10^{-4}$ (4·5)	$3{\cdot}1\times10^{-3}$ (4·7)	$4{\cdot}5\times10^{-4}$ (5·2)	$1{\cdot}6\times10^{-3}$ (3·0) $1{\cdot}2\times10^{-3}$ (3·0)	$>2{\cdot}2\times10^{-1}$ $>3{\cdot}6\times10^{-1}$
p-nitrophenol pK = 7·2	$3{\cdot}5\times10^{-4}$ (3·5) $3{\cdot}5\times10^{-4}$ (4·1) $3{\cdot}3\times10^{-4}$ (4·7) $3{\cdot}7\times10^{-4}$ (5·2)	$1{\cdot}3\times10^{-3}$ (2·7) $1{\cdot}4\times10^{-3}$ (4·7)	$6{\cdot}8\times10^{-5}$ (5·2)	$2{\cdot}5\times10^{-4}$ (3·0)	$1{\cdot}2\times10^{-2}$ $7{\cdot}9\times10^{-3}$
2:4-dinitrophenol pK = 4·0	$6{\cdot}8\times10^{-6}$ (3·2) $\mathbf{5{\cdot}4\times10^{-6}}$ (<pK)	$3{\cdot}5\times10^{-5}$ (2·4)	$8{\cdot}0\times10^{-6}$ (5·4) $\mathbf{5{\cdot}0\times10^{-7}}$ (<pK)	$1{\cdot}3\times10^{-5}$ (3·0) $\mathbf{1{\cdot}2\times10^{-5}}$ (<pK)	$1{\cdot}5\times10^{-2}$ $\mathbf{4{\cdot}7\times10^{-4}}$ (<pK)

double nitration in increasing the degree of toxicity. Phenol is always the least toxic compound; the substitution of a single nitro group in the more toxic *para* position on average causes a 44-fold increase in activity. A second nitration raises the level of toxicity by 56 times.

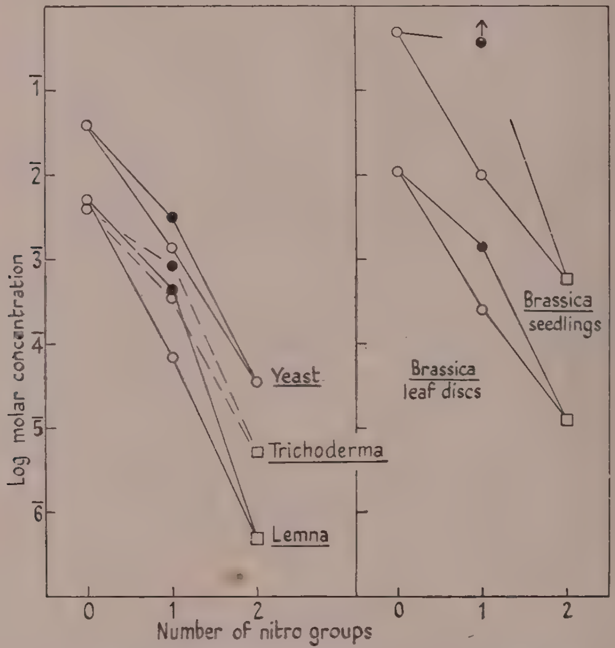

FIG. 1. The molar concentrations of phenol, *o*- and *p*-nitrophenol, and 2:4-dinitrophenol required at pH levels below pK to halve the growth rate of *Trichoderma viride* and of *Lemna minor*, to halve the respiration of yeast and of infiltrated leaf disks of *Brassica alba*, and to give a 50% mortality of seedlings of *B. alba*. Circles indicate measured concentrations, the squares being estimates of the concentrations that would be required at a pH below pK. *Ortho*-nitrophenol is shown as a black point to distinguish it from the *para* isomer.

One striking feature of the results is the failure of *o*-nitrophenol to cause appreciable injury in the spraying experiments. Thus the highest spray concentration used in one experiment, namely 5 per cent. (equivalent to $3{\cdot}6 \times 10^{-1}$ M.), killed no more than 3 per cent. of the seedlings. On the other hand, the toxicity of *p*-nitrophenol always fell within the expected range midway between the toxicity of phenol and that of 2:4-dinitrophenol. It was first thought that this difference might be ascribed to the very limited solubility of *o*-nitrophenol in water, coupled with the difficulty of making suspensions. It seemed possible that the suspension might have separated out during the spraying operations so that the droplets deposited on the plant were much weaker than the original spray mixture. An experiment was therefore under-ten in which *ortho-* and *para*-nitrophenols were sprayed on to seedlings as te solutions in acetone. The results were similar to those previously obtained. The strongest solution of *o*-nitrophenol employed, 1 per cent. ($7{\cdot}2\times$ 1^{-2} M.), only gave a 13 per cent. mortality, while just under 0·1 per cent. (2×10^{-3} M.) *p*-nitrophenol was sufficient to bring about a 50 per cent. kill. though acetone alone caused some immediate injury it did not cause the eath of any plants. On the basis of these experiments it is suggested that the fference in toxicity should be attributed to the greater volatility of the *ortho* omer. The plants were sprayed and then left in the open for about a week fore the final counts were made, and during this period there may well have en a significant loss of *o*-nitrophenol from the leaf surfaces.

When the various compounds were received from the manufacturers information was obtained on their degree of purity and melting-points. The picric id was listed as 'titration 100 per cent.' and all the different tests were rried out with this material. Some time after the experimental work had een completed the occasion arose, in connexion with other researches, to rry out some further experiments with *L. minor* using in this instance a new atch of picric acid. Although the experimental conditions were somewhat ifferent, it was clear from the new data that the two batches differed greatly n toxicity, and the conclusion was reached that at least one of the batches ontained a biologically active impurity. It was decided to attempt a purification of the second batch because of the much larger amount available and then repeat some of the original tests. However, repeated washing with water nd dilute hydrochloric acid followed by precipitation from solution with ydrochloric acid had no effect on the melting-point and it therefore seemed hat the second batch was a pure product.

Only a very small quantity of the first batch was available and a comparison f the two samples could only be made in the yeast respiration test. The results howed that the second batch was about 10 times less toxic than the first, the oncentration required to halve the respiration rate at pH 3 being $1{\cdot}3\times10^{-2}$ M. As the exact conditions of the original tests with *L. minor* could not be repeated it was decided to compare the toxicity levels of 2:4-dinitrophenol and he purified second batch of picric acid. The compounds were added to a Steinberg (1941) culture solution containing double the normal phosphate evel, and the pH adjusted to 5·1. The plants were grown under a light ntensity of 315 foot-candles and at a temperature of 25° C. The concentrations of dinitrophenol required to halve the growth rate showed reasonable agreement—$9{\cdot}8\times10^{-6}$ M. as against the initial $8{\cdot}0\times10^{-6}$ M., but the results for the two batches of picric acid were markedly different. For the original batch the required concentration was $2{\cdot}4\times10^{-4}$ M. and for the purified material $7{\cdot}6\times10^{-3}$ M.

Little information is available on the effect of pH on the toxicity of an acid as strong as picric acid (pK = 0·8), although preliminary experiments have shown that with a change of pH from 4·6 to 5·6 there is a change in the toxicity to *L. minor*. Even so, it is doubtful whether a correction should be applied to the measured value of toxicity as was done with the dinitrophenol results. However, whether such a correction is applied or not, it remains the case that picric acid is less toxic to yeast and *L. minor* than dinitrophenol.

The relative toxicity of other benzene derivatives and their nitro compounds. In investigating the toxicities of nitro derivatives of anisole, phenetole, toluene, and nitrobenzene, only the *T. viride* test was employed and the toxicity of each compound was normally measured over a range of pH. However, as these compounds are all non-electrolytes, pH has no effect on the magnitude of the response (Simon and Blackman, 1949). In Fig. 2 the logarithms of the mean concentrations required to halve the growth rate have been plotted against the degree of nitration. It will be noted that no value is given for the toxicity of toluene, since this compound and benzene were both non-toxic in saturated solutions. In the nitrobenzene series a value is given for the toxicity of trinitrobenzene, but it must be pointed out that the symmetrical 1:3:5 isomer was used, instead of the 1:2:4 isomer which would correspond more closely to the other di-substituted compounds, such as 2:4-dinitrotoluene and 2:4-dinitroanisole.

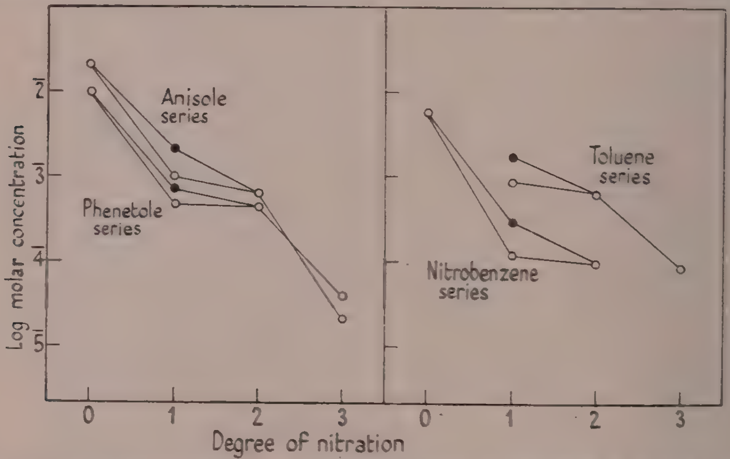

FIG. 2. The effects of the degree of nitration on the concentrations of several substituted benzenes required to halve the growth rate of *T. viride*. In each series 0 represents the parent materials, i.e. anisole, phenetole, toluene, and nitrobenzene; 1, the *ortho*- and *para*-nitro derivatives, of which the *ortho* is shown as a black point; 2, the 2:4-dinitro derivative of anisole, phenetole, and toluene; also 1:3:5-trinitro benzene (see text); 3, the 2:4:6-trinitro derivative.

The results with the different series of compounds are in close agreement, but the general trend is clearly differentiated from the phenol series. The change from the parent material to the *p*-nitro derivative results, on the average, in a 30-fold increase in toxicity, but the second nitration, instead of bringing about a similar increase, only results in a slight increase of 1·3 times. Again, in contrast to the phenol series, the third nitration causes the toxicity to increase by a factor of 16.

DISCUSSION

The present investigations have demonstrated that as long as allowance is ade for the pH factor, the change of activity in the nitrophenol series is emarkably consistent irrespective of the test organism and the nature of the iological response recorded. Considering the magnitude of the changes in he equi-effective concentration resulting from the addition of successive itro groups it is perhaps not surprising that there is some small variation etween the different methods employed for assessment. For instance, the oxicity of phenol to *T. viride* is less than would be expected on the basis of he data for *L. minor*, yeast, or the infiltrated leaf disks. Again, it was found hat *o*-nitrophenol is only very slightly toxic to seedlings of *B. alba*, but it has lready been emphasized that differences in volatility, which would not perate in the other methods, must be taken into account.

It is evident that the relationship found between structure and toxicity vould have been different if the pH factor had been ignored, and from this oint of view it is of interest to re-examine the data for the relative toxicities f nitrophenols obtained by previous investigators. For example, Plantefol 1922) working with spores of *Aspergillus niger* measured the concentration equired to inhibit germination in an acid medium, and on the whole his esults are in line with the present findings, dinitrophenol being 10 times as oxic as *p*-nitrophenol (see Fig. 3). Krahl and Clowes (1938) conducted a eries of tests with *Echinoderm* eggs in solutions of pH 7·5, and their results also given in Fig. 3) show that, at this pH, dinitrophenol is only 4·4 times more toxic than *p*-nitrophenol. With yet more alkaline solutions it would be anticipated that the toxicity of dinitrophenol would become less than that of *p*-nitrophenol. This reversal in toxicity is illustrated in Fig. 3 by the results of Woodward *et al.* (1934), who made up their test solutions of dinitrophenol and picric acid in 1 per cent. sodium carbonate, which has a pH of about 9, while phenol and *p*-nitrophenol were tested at pH 5·3.

The value which Woodward *et al.* (1934) obtained for the toxicity of picric acid diverges from the general trend, and in view of the variation in toxicity between samples of picric acid found in the present investigation it is of interest to comment on the errors that are likely to arise through traces of impurity. If the contaminant is itself a weak acid, a very small amount may cause an appreciable increase in the over-all toxicity in acid media. For example, in the *L. minor* experiment the discrepancy between the two batches of picric acid could be accounted for if one sample contained less than 1 part in 2,000 of 2:4-dinitrophenol. This source of error will be less important where the pH factor does not operate, as in spray applications to whole plants, and under these conditions it has been reported that picric acid is almost completely ineffective for the control of annual weeds (Chabrolin, 1943; Wain, 1944), a finding which was confirmed in the present experiments.

Although only one test organism, *T. viride*, was used to assess the relative toxicity of the remaining compounds, the general agreement over all tests for the nitrophenol series would suggest that the results with the remaining compounds are not specific to *T. viride*. For the nitrobenzene series, Tattersfield *et al.* (1925) and Bushland (1940) have undertaken parallel tests on insects, and their results show a similar trend although the differences in toxicity between compounds were smaller than those observed in the present investigations. In this connexion, it may be noted that questions of the distribution of the toxicant between the phase in which it is applied and the various internal phases of the test organism are likely to be more complex in a large and highly differentiated organism, such as an insect, than in a fungal mycelium.

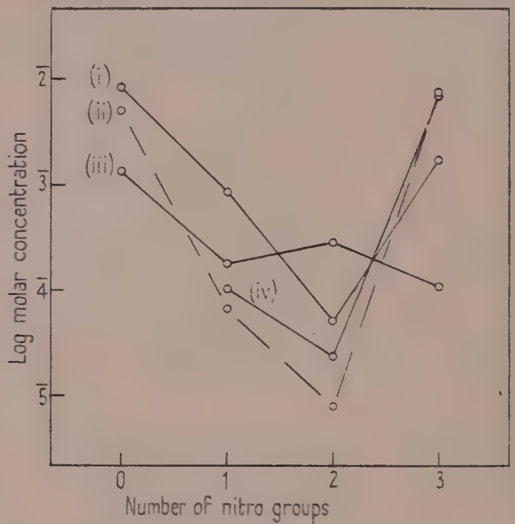

FIG. 3. The concentrations of phenol, *p*-nitrophenol, 2:4-dinitrophenol, and 2:4:6-trinitrophenol required (i) to stop the germination of spores of *Aspergillus niger* (data of Plantefol, 1922), (ii) to halve the rate of frond multiplication of *Lemna minor* at pH 5·1 to 5·4, (iii) to inhibit the growth of *Monilia tropicalis* (data of Woodward *et al.*, 1934), and (iv) to halve the rate of cell division of fertilized eggs of *Arbacia punctulata* (data of Krahl and Clowes, 1938).

One further aspect of the relationship between structure and toxicity remains to be discussed—*ortho para* isomerism. For the mononitroanisoles and phenetoles the *para* compound is slightly more toxic than the *ortho*. In the present experiments the *p*-nitro derivatives were consistently found to be more toxic than the *o*-nitro (Figs. 1 and 2). The same result has been obtained by other workers with bacteria (Caius *et al.*, 1927; review by Suter, 1941), fungi (Plantefol, 1922; Blanck, 1933), insects (Tattersfield, 1927), and seedlings (Chabrolin, 1943). This difference in toxicity can perhaps be related to the fact that *o*-nitrophenol is less polar an *p*-nitrophenol, having a higher volatility and a lower solubility (Sidg- ick, 1945, p. 268).

That progressive nitration leads to a marked increase in toxicity is a feature ommon to the four groups of compounds, and the question arises whether is general trend can be linked with corresponding changes in physical or hemical properties. As long ago as 1893, Richet observed that toxicity is ften inversely related to solubility, and since then other workers have shown, articularly in homologous series, that there is a relationship between toxicity nd physical properties. In Fig. 4 the concentrations of the nitrophenols, itrotoluenes, and nitrobenzenes required to halve the growth rate of *T. viride* ave been plotted on a logarithmic scale together with the corresponding olubilities. For this purpose solubility values have been taken from the iterature, although it is appreciated that the accuracy of some of the determinations is open to question. In a few cases different authorities give different values, and in addition it must be noted that the present toxicity measurements were made at 25° C. and in the presence of a phosphate buffer, while published solubility values refer to various temperatures between 15° C. and 30° C. and to the solubility in distilled water. It is clear that the pH of the solution affects the solubility as well as the toxicity of weak acids of low pK, but solubility measurements for di- and trinitrophenols at pH levels approaching pK have not been found in the literature. However, the solubility of 2:4-dinitrophenol has been measured in this laboratory by R. S. Bruce at 25° C. in 10 g./l. phosphate at pH 3.

It will be observed from Fig. 4 that in the toluene series the changes in toxicity and changes in solubility follow very similar trends with increasing degrees of nitration and moreover that the toxic concentrations are only a little less than the solubility values. The nitrophenols and nitrobenzenes, on the other hand, show the standard toxic effect in solutions which are far from saturated, and the relationship between toxicity and solubility is less evident, the solubilities of the *ortho* and *para* mono-substituted compounds being in the reverse order to their relative toxicities.

Ferguson (1939, 1948) on the basis of thermodynamic considerations has postulated that when there are conditions of equilibrium between the toxic substance in the external medium and at the site of action within the cell, then the partial molal free energy or 'activity' is a measure of the intrinsic toxicity at the biophase. For substances which are sparingly soluble in water Ferguson concludes that as a first approximation the activity can be expressed as the ratio of the external concentration to the solubility.

Examining the variation in activity for a wide range of substances Ferguson distinguishes two types of toxic action—physical and chemical. When a distribution equilibrium has been set up between all the phases concerned and the physiological effect is a function of the external concentration, the primary reaction is a physical one, such as narcosis, where the action is that of whole molecules. Such a primary process is reversible, and where there are irreversible effects, such as death, these must be looked upon as secondary effects. Chemical toxicity arises when chemical reactivity predominates and the toxic compound is 'being used up appreciably by interfering chemically with reaction sequences within the organism'. There is no true phase-equilibrium, although physical factors of distribution may affect the external concentration needed to bring about the toxic effect.

Ferguson himself emphasized that the boundary between the two types of toxic action is not a sharp one, but he is of the opinion that in general an activity value of 0·1–1·0 denotes that the action is largely physical, while a lower figure indicates a chemical action. On the other hand, Albert (1951) questions whether such a distinction can be made since chemical reactions can no longer be regarded as restricted to the making and breaking of ionic and covalent bonds since hydrogen-bonds, ion-dipole bonds, and van der Waals's forces may all play a part. Indeed, Albert considers that under the conditions of equilibrium associated with a reversible toxic effect, it is unlikely that covalent bonds will often be involved because of the great strength with which they bind molecules together.

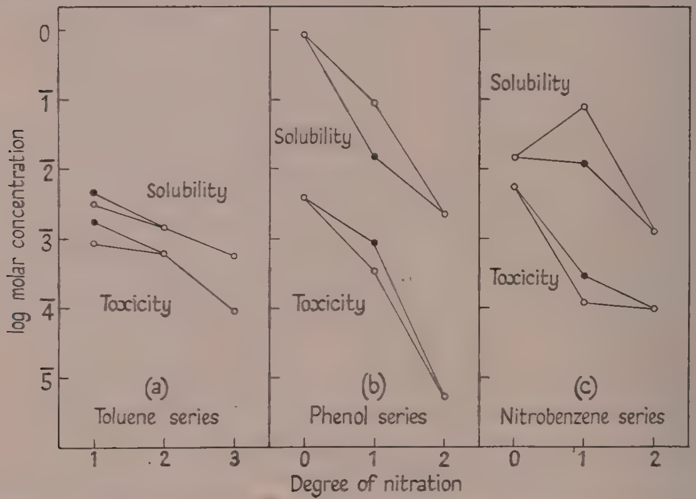

FIG. 4. Comparison of the solubilities of various nitro compounds with the concentrations required to halve the growth rate of *T. viride*. The compounds shown in (a) are *o*- and *p*-nitrotoluene, 2:4-dinitrotoluene, 2:4:6-trinitrotoluene; in (b) phenol, *o*- and *p*-nitrophenol, 2:4-dinitrophenol; and in (c) nitrobenzene, *o*- and *p*-dinitrobenzene and 1:3:5-trinitrobenzene. Black points indicate the *ortho* isomers.

A distinction is made by Albert between 'structurally non-specific' and 'structurally specific' substances. Structurally non-specific compounds are those in which a common biological effect is not dependent on the presence or absence of particular chemical groups in the molecule; conversely, structurally specific substances are those in which a small alteration in structure is likely to produce a major change in biological activity. Structurally non-specific substances can be regarded as 'acting simply as foreign bodies' which accumulate in vital *loci* within cells and thereby interfere with the reaction sequence of enzyme systems.

Albert points out that the Ferguson principle as defined by 'substances which are present at the same proportional saturation in a given medium have the same degree of biological action' seems to hold for all the structurally non-specific substances so far investigated by various workers. From these findings it follows that if in a biological test of toxicity the activity ('Ferguson value') is first determined for a number of substances known to be structurally non-specific, then for new compounds it is possible to assess whether they are also in the same class. Alternatively, when the value shows a wide variation within a group of allied compounds, it can be concluded that toxicity is related to chemical structure.

TABLE II

he activity of nitro compounds determined from toxicity measurements with Trichoderma viride

	Molar concentration required for 50% inhibition of growth of *Trichoderma viride* (*a*)	Solubility (molar) (*b*)	Activity = *a*/*b*
henol	$4{\cdot}0 \times 10^{-3}$	$8{\cdot}7 \times 10^{-1}$	0·0046
-nitrophenol	$8{\cdot}7 \times 10^{-4}$	$1{\cdot}5 \times 10^{-2}$	0·058
-nitrophenol	$3{\cdot}5 \times 10^{-4}$	$9{\cdot}3 \times 10^{-2}$	0·0038
:4-dinitrophenol	$5{\cdot}4 \times 10^{-6}$	$2{\cdot}2 \times 10^{-3}$*	0·0025
-nitroanisole	$2{\cdot}3 \times 10^{-3}$	$1{\cdot}1 \times 10^{-2}$	0·21
-nitroanisole	$1{\cdot}0 \times 10^{-3}$	$3{\cdot}9 \times 10^{-3}$	0·26
:4-dinitroanisole	$6{\cdot}5 \times 10^{-4}$	$8{\cdot}1 \times 10^{-4}$	0·80
-nitrotoluene	$1{\cdot}8 \times 10^{-3}$	$4{\cdot}7 \times 10^{-3}$	0·38
-nitrotoluene	$8{\cdot}4 \times 10^{-4}$	$3{\cdot}2 \times 10^{-3}$	0·26
:4-dinitrotoluene	$6{\cdot}3 \times 10^{-4}$	$1{\cdot}5 \times 10^{-3}$	0·42
:4:6-trinitrotoluene	$8{\cdot}9 \times 10^{-5}$	$5{\cdot}7 \times 10^{-4}$	0·16
Nitrobenzene	$5{\cdot}6 \times 10^{-3}$	$1{\cdot}5 \times 10^{-2}$	0·37
-dinitrobenzene	$3{\cdot}0 \times 10^{-4}$	$1{\cdot}2 \times 10^{-2}$	0·025
-dinitrobenzene	$1{\cdot}2 \times 10^{-4}$	$7{\cdot}9 \times 10^{-2}$	0·0015
:3:5-trinitrobenzene	$9{\cdot}9 \times 10^{-5}$	$1{\cdot}3 \times 10^{-3}$	0·076

* Value determined by Mr. R. S. Bruce by a colorimetric method. Other values of solubility taken from Seidell (1941).

The concepts of Ferguson and Albert may now be applied to the results of the present investigation; the most comprehensive data are available from the experiments with *T. viride* (Table II). For both the nitroanisoles and the nitrotoluenes the activity values are relatively high and of the same order, suggesting that the action of these compounds is structurally non-specific. Admittedly, more compounds within each group will have to be tested before a firm conclusion can be reached, although it may be noted that there is evidence that ethers as a class are structurally non-specific (Albert, 1951). On the other hand, there is an appreciable difference between the activities of nitrobenzene and di- and trinitrobenzene indicating structural specificity.

Again, the very low values for the phenol series suggests that they too are structurally specific.

From Fig. 1 it is apparent that the concentrations of nitrophenols required to bring about the responses recorded in the different techniques of assessment are widely divergent and these differences will in turn affect the magnitude of the activity. Since the concentrations required to halve the growth rate of *T. viride* and *L. minor* were markedly less than those needed to reduce the oxygen consumption of yeast and the leaf disks of *B. alba* by 50 per cent., the activity values are also lower (see Table III). The general trend for activity to change between phenol and the nitrophenols indicates that the variation in toxicity is related to chemical structure.

TABLE III

The activity of phenol and nitrophenols determined with several test organisms. The activities have been calculated from the data for toxicity at low pH levels, cited in Table I

Test response . .	Phenol	*o*-nitrophenol	*p*-nitrophenol	2:4-dinitrophenol
Yeast respiration . .	0·044	0·21	0·015	0·016
Respiration of infiltrated *Brassica* leaf disks .	0·013	0·093	0·0027	0·0057
Trichoderma viride growth .	0·0046	0·058	0·0038	0·0025
Lemna minor growth .	0·0059	0·030	0·00073	0·00023

Some tentative conclusions can be reached as to why the concentrations of nitrophenols needed to halve the respiration rate are greater than those causing a 50 per cent. inhibition of growth. It has been established for a wide range of organisms that as the concentration of a dinitrophenol is increased there is first a rise in the respiration rate followed by a depression at higher concentrations. It is also known that a concentration of a dinitrophenol sufficient to stimulate respiration can interfere with many synthetic processes such as the formation of new enzymes, cell division, and growth. The effects of mononitro- and dinitrophenols on the division of fertilized *Echinoderm* eggs and on their respiration rate were investigated in detail by Clowes and Krahl (1936), and it can readily be seen from their figures that inhibition of cell division is caused by concentrations which stimulate the respiration while considerably higher concentrations are demanded to bring about a 50 per cent. reduction in the respiration rate. Experiments by one of us (E. W. S.) on the effects of dinitrophenols on respiration and on oxidative assimilation in yeast reveal similar relationships; anabolic processes are more susceptible to dinitrophenols than is respiration.

Lastly, there is one further aspect on which comment should be made, namely, the apparent conditions of equilibrium between the external concentration and the toxic effect observed in each series. Both in the respiration experiments, which lasted only 120 minutes, and in the *T. viride* and *L. minor* tests, which covered a period of days, the magnitude of the reduction in spiration or growth rate brought about by a particular concentration remained constant during the experimental period.

During the process of cell initiation and differentiation there will be a hanging pattern in the cell constituents and in the enzyme systems. On this asis, it can be put forward that during the course of differentiation (i) the egree of accumulation of a toxic substance within the cell may vary, (ii) the tes of toxic action may change, and (iii) the numbers and rate of formation f reaction centres may also vary. In an actively growing organism these factors lone or in combination may contribute to the toxic effect.

In the respiration experiments the conditions were such as to arrest cell ivision, but this was not so with *L. minor*. With non-lethal concentrations of he nitrophenols daughter fronds continued to be produced, while there was an overall gain in weight during the experimental period. The reductions in he growth rate must have involved a depression in meristematic activity, and ince other experiments have shown that such a reduction in the frond multiplication rate does not bring about an appreciable decrease in frond size, it follows that the decrease in meristematic activity must have been matched by a reduction in the rate of extension growth and subsequent differentiation. It would therefore appear that the toxic action of the nitrophenols is not closely associated with the phase of cell differentiation, i.e. that they act at some vital point in the chain of fundamental processes which are essential to the metabolism of all the cells. This supposition is supported by the results of many previous investigations. It has already been emphasized that the effects on respiration are similar for a wide range of tissues and organisms, and it is now generally agreed that the common biological effects are due to interference with the transfer of energy.

It is of interest to contrast the effects of 2:4-dichlorophenoxyacetic acid on the growth of *L. minor*, for in this instance the depression of the growth rate is cumulative and there is a progressive reduction in frond size (Blackman, 1952). These trends suggest that unlike the nitrophenols, dichlorophenoxyacetic acid is most toxic at some particular phase of cell division or differentiation.

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Microbiological Synthesis of Fat

THE FORMATION OF FAT FROM SUCROSE

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SUMMARY

1. Forty-three strains of moulds, selected from ten species, were examined to ascertain their capabilities as producers of fat, when grown in four different solutions of nutrient inorganic salts together with sucrose. The three highest yields of felt were given by *Penicillium javanicum* van Beyma, *P. Soppi* Zaleski, and *Aspergillus nidulans* Eidam, in that order. The fat content on felt weight was maximal at 34·8 per cent. with *P. Soppi*, at 28·4 per cent. with *Fusarium lini* (1), and at 25·8 per cent. with *A. nidulans*; on sugar utilized it was maximal at 11·4 per cent. with *P. Soppi*, at 7·9 per cent. with *A. nidulans*, and at 5·6 per cent. with *F. lini* (1).

2. Other moulds considered worthy of further trial were *P. spinulosum*, *P. aurantiobrunneum*, *F. graminearum*, *A. flavus*, *F. oxysporum*, *A. clavatus*, and *P. oxalicum*.

PREVIOUSLY most of the studies of the production of fat by moulds have been carried out with glucose as the source of energy and only a few comparisons have been made between glucose and sucrose as carbon substrates. f fats are to be prepared by the industrial utilization of moulds, it is obvious that on grounds of economy carbon sources such as cellulose waste and molasses must also be considered. Since sucrose is the major carbohydrate onstituent of molasses it was decided, prior to working with the latter, to scertain the behaviour of different moulds towards pure sucrose. Recently Woodbine, Gregory, and Walker (1951) reported the results obtained during a preliminary survey of the potentialities of 43 strains of 10 species of moulds s producers of fat in media containing mineral salts and glucose. The results obtained when growing these same 43 strains on the same salt solutions, with sucrose substituted for glucose, are now communicated.

EXPERIMENTAL

Strains. The strains of mould used in these experiments were all subcultures of those used by Woodbine, Gregory, and Walker (1951). In this former work two strains of *Fusarium lini* Bolley which were used were distinguished by the numbers (1) and (2). *F. lini* Bolley (1) was obtained from the Baarn Collection and *F. lini* Bolley (2) was obtained through the courtesy of Professor F. F. Nord.

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Maintenance. The strains were maintained on brewery malt wort, unhoppe and containing 2 per cent. agar, prepared according to the method of Tosi and Walker (1944). Subcultures were taken at intervals of approximatel 6 weeks.

Media. Four media were used, sucrose forming the carbon source in a cases. Medium A contained the mixture of salts employed by Prill, Wencl and Peterson (1935) together with sucrose (20 per cent. w/v) and the pl value was 6·8. Medium B2 was that of Lockwood *et al.* (1934; see also War *et al.*, 1935) with sucrose (22·5 per cent. w/v) and with addition of corn-stee liquor (0·5 ml./100 ml.), and the pH value was 5·7. The basal salts of mediu C were those of Czapek-Dox as modified by Raistrick and Vincent (1948 the sucrose content was 5 per cent. (w/v), and the pH value was adjusted t 6·1. Medium D was based on that of Bernhauer *et al.* (1948), but containe 0·1 per cent. of ammonium sulphate in place of 0·01 per cent. (w/v) of th salt as used by these authors. The sucrose content was 1 per cent. (w/v) an the pH value was 5·1. The reagents were all B.D.H. Analar grade, and th sucrose was the commercial product of high purity. Full details of these bas salt mixtures were given in the communication of Woodbine *et al.* (1951).

Inoculation. Each mould was inoculated from a 4–5 days old culture. Aft incubation, in all cases at 25° for 14 days, the felts were harvested, washe and dried.

TABLE I

Moulds giving the highest yields of felt and fat when grown on medium A at 25° (
for 14 days

Mean felt weight (g.)		Fat (g./100 g. dry felt)		Fat (g./100 g. sugar utilized)	
P. javanicum	2·167 (±0·217)	*F. graminearum*	10·30	*Z. Moelleri*	2·
P. Soppi	1·886 (±0·018)	*F. oxysporum*	7·74	*P. javanicum*	2·
A. nidulans	1·864 (±0·043)	*Rh. oryzae*	7·27	*F. graminearum*	2·
P. piscarium	1·799 (±0·104)	*F. lycopersici*	7·12	*P. spinulosum*	2·
P. aurantiobrunneum	1·605 (±0·086)	*U. zeae*	7·02	*P. aurantiobrunneum*	1·
P. flavocinereum	1·586 (±0·173)	*P. spinulosum*	6·79	*M. mucedo*	1·
P. spinulosum	1·531 (±0·131)	*F. lini* (2)	6·77	*Rh. nigricans*	1·
F. lini (1)	1·403 (±0·083)	*Paecilomyces varioti*	6·43	*Rh. oryzae*	1·
C. herbarum	1·361 (±0·089)	*P. aurantiobrunneum*	6·07	*Paecilomyces varioti*	1·
A. niger	1·344 (±0·054)	*M. spinosus*	5·61	*U. zeae*	1·
P. luteum	1·330 (±0·218)	*F. lini* (1)	5·53	*P. flavocinereum*	1·
P. purpurogenum	1·300 (±0·112)	*P. javanicum*	5·41	*F. lini* (1)	1·

Estimation of fat was performed as already described by Woodbine *et al.* 951).

Sugar estimation. To the combined solutions and washings from each batch f five flasks 5 ml. of hydrochloric acid (sp.gr. 1·189) were added to hydrolyse nchanged sucrose. The mixture was made up to a given volume and allowed stand for 1 week before analysis by the method of Lane and Eynon (1923).

RESULTS

The twelve best results obtained from each medium are shown in Tables to IV, other results being omitted to conserve space. Table I shows the esults from medium A after 14 days incubation at 25° C., the moulds being sted in order of decreasing mean felt weight. The standard deviations are ited in brackets immediately below the weights. G. fat per 100 g. dry felt nd g. fat per 100 g. sugar utilized are also given in each case. Results from edia B2, C, and D are set out similarly in their respective Tables, II, III, nd IV.

TABLE II

oulds giving the highest yields of felt and fat when grown on medium B2 at 25° C. for 14 days

Mean felt weight (g.)		Fat (g./100 g. dry felt)		Fat (g./100 g. sugar utilized)	
P. Soppi	1·549 (±0·225)	*P. Soppi*	29·68	*P. Soppi*	9·24
P. javanicum	1·285 (±0·098)	*F. lini* (1)	28·36	*M. albo-ater*	9·23
P. aurantiobrunneum	1·160 (±0·041)	*A. nidulans*	25·79	*A. nidulans*	7·91
P. spinulosum	1·122 (±0·026)	*M. albo-ater*	21·81	*F. lini* (1)	5·61
P. piscarium	1·107 (±0·112)	*Z. Moelleri*	18·44	*Z. Moelleri*	5·20
A. nidulans	1·092 (±0·133)	*U. zeae*	18·33	*M. Ramannianus*	5·14
P. flavocinereum	1·024 (±0·055)	*P. spinulosum*	17·00	*P. spinulosum*	4·22
A. flavipes	0·972 (±0·095)	*Rh. nigricans*	15·56	*U. zeae*	4·07
P. oxalicum	0·882 (±0·035)	*A. clavatus*	14·97	*Mortierella* sp.	3·16
A. clavatus	0·844 (±0·022)	*F. oxysporum*	13·71	*A. clavatus*	2·72
F. lini (1)	0·797 (±0·104)	*A. flavus*	13·11	*Rh. nigricans*	2·64
A. flavus	0·752 (±0·049)	*M. spinosus*	12·00	*A. flavus*	2·59

DISCUSSION

At the lower concentrations of sugar (Tables III and IV) the percentage values found for the fat contents of the dry felts are seen to lie between limits nearly the same as those comprising the corresponding values found for the felts grown on medium B2 (Table II). It will be observed, however, th moulds which produced good results on one medium behaved similarly o one or more of the other media only in a few cases. Medium A proved dis appointing, for although it permitted formation of the heaviest felts, thes were relatively low in fat content. Low felt weights had been anticipated fro the flasks containing media C and D, respectively, because these had lo concentrations of sucrose, but such low yields of felt would have been offs had the cultures from these flasks shown high ratios of g. fat to 100 g. sugar utilized. This ratio was lower on the average, however, in the felts fro medium C and medium D, than in the felts from medium B2.

TABLE III

Moulds giving the highest yields of felt and fat when grown on medium C 25° C. for 14 days

Mean felt weight (g.)		Fat (g./100 g. dry felt)		Fat (g./100 g. sugar utilized)	
P. Soppi	0·406 (±0·021)	*P. Soppi*	34·78	*P. Soppi*	11·
P. roqueforti	0·371 (±0·041)	*U. zeae*	27·83	*T. viride* (2)	10·
P. oxalicum	0·356 (±0·017)	*F. lini* (1)	26·35	*T. viride* (1)	6·
P. javanicum	0·347 (±0·011)	*F. graminearum*	24·07	*A. minutus*	4·
P. piscarium	0·332 (±0·017)	*T. viride* (2)	24·00	*P. oxalicum*	4·1
P. spinulosum	0·297 (±0·008)	*Rh. nigricans*	18·33	*U. zeae*	3·5
P. aurantiobrunneum	0·276 (±0·045)	*M. plumbeus*	17·88	*Rh. nigricans*	3·4
A. minutus	0·258 (±0·122)	*A. clavatus*	14·20	*P. aurantiobrunneum*	3·1
A. flavus	0·255 (±0·013)	*F. oxysporum*	13·90	*F. lini* (1)	2·9
P. varioti	0·210 (±0·109)	*A. minutus*	13·56	*F. graminearum*	2·8
A. flavipes	0·208 (±0·009)	*T. viride* (1)	13·33	*M. plumbeus*	2·8
A. niger	0·188 (±0·031)	*P. flavocinereum*	12·50	*A. flavipes*	2·8

Finally, although the felts grown on medium B2 were for the most part less weight than those from medium A, the actual weight of fat produced fro medium B2 was greater than that yielded by the cultures from medium hence medium B2 (or modifications of the same) appears to be preferable medium A. On medium B2 *P. Soppi* was outstanding with a yield of 2·30 of fat from 5 felts having a total weight of 7·75 g. The yield of fat was th 29·68 per cent. on dry felt and on the sugar utilized it was 9·24 per cen *F. lini* (1) also gave good results on this medium.

The tabulated data indicate that the following eleven moulds show promising overall behaviour as producers of fat from sucrose: *P. Soppi*, *F. lini* (1), *. spinulosum*, *P. aurantiobrunneum*, *A. nidulans*, *F. graminearum*, *P. javanicum*, *A. flavus*, *F. oxysporum*, *A. clavatus*, and *P. oxalicum*.

It is interesting to note that *A. nidulans*, *P. spinulosum*, and *P. javanicum* ere among eight moulds judged to be of promise on the results of the experiments on fat formation from glucose media (Woodbine *et al.*, 1951).

TABLE IV

Moulds giving the highest yields of felt and fat when grown on medium D at 25° C. for 14 days

Mean felt weight (g.)		Fat (g./100 g. dry felt)		Fat (g./100 g. sugar utilized)	
P. Soppi	0·068 (±0·013)	*F. lini* (2)	32·22	*A. insuetus*	5·83
A. niger	0·064 (±0·010)	*F. oxysporum*	24·44	*F. lini* (2)	4·60
C. herbarum	0·058 (±0·006)	*F. graminearum*	24·44	*F. oxysporum*	3·50
P. javanicum	0·057 (±0·012)	*F. bulbigenum* (2)	20·00	*P. Soppi*	3·41
P. varioti	0·056 (±0·002)	*F. bulbigenum* (3)	16·84	*A. nidulans*	3·33
P. aurantiobrunneum	0·055 (±0·006)	*U. zeae*	16·66	*A. flavipes*	3·33
P. oxalicum	0·055 (±0·004)	*F. lycopersici*	16·15	*F. graminearum*	2·89
P. purpurogenum	0·054 (±0·005)	*P. luteum*	16·00	*F. bulbigenum* (2)	2·87
P. piscarium	0·053 (±0·003)	*F. lini* (1)	15·00	*A. flavus*	2·67
M. circinelloides (1)	0·051 (±0·004)	*T. viride* (2)	13·33	*M. circinelloides* (1)	2·62
A. flavus	0·051 (±0·007)	*M. circinelloides* (1)	12·94	*U. zeae*	2·56
A. clavatus	0·050 (±0·006)	*A. nidulans*	12·94	*F. bulbigenum* (3)	2·54

ACKNOWLEDGEMENT

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The Activity of Organic Acids as Germination Inhibitors and its Relation to pH

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SUMMARY

The germination inhibitory activity of organic acids related to coumarin was investigated. The activity was found to be unrelated to the nature of the side chain carrying the carboxyl group. Little or no relation between structure and activity existed. pH was found to be of secondary importance on the activity of the acids. Synergism between buffers and the acids exists in some cases. Moreover, the use of buffers in studying germination inhibition was found to be unnecessary, due to the powerful buffering action of the seeds, and impermissible due to very marked secondary effects of the buffers themselves and their effect on germination.

INTRODUCTION

N a continuation of the study of germination inhibitors related to coumarin Mayer and Evenari, 1952) a number of organic acids were examined. This as done in order to obtain further information on the relation between tructure and activity of germination inhibitors. At the same time the pportunity was taken to reinvestigate certain claims about the activity of errulic and caffeic acids as inhibitors (Akkerman and Veldstra, 1947) and egarding the relative activities of *cis*- and *trans*-cinnamic acids (Veldstra, 943–5).

As the comparison of the activity of organic acids may involve pH effects, s has been pointed out by Blackman, Holly, and Roberts (1949), it was lecided to reinvestigate the entire question of the effect of pH on the activity f inhibitors and particularly to find out what changes occurred in pH during he process of germination. For this purpose various buffers were studied and ome previous findings regarding the synergistic effects of buffers were econfirmed (Levari, Mayer, and Evenari, 1952).

METHODS

The substances were tested on lettuce seeds, variety 'Progress' (kindly upplied by Pieters–Wheeler)[1] by the methods previously described (Mayer nd Evenari, 1952; Levari, Mayer and Evenari, 1952). From the results the nhibition index (I.I.) of the substances was calculated. The I.I. is the molarity f coumarin giving 50 per cent. inhibition divided by the molarity of the ubstance giving 50 per cent. inhibition. The pH was measured using a glass

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electrode, both at the beginning of the experiment and on solution left in th Petri dishes after a germination period of 48 hours.

The results are given as the means of a number of replicates. The graph showing the changes of pH during the experiment consist of two parts. Th one shows the curve of germination percentage with varying concentratio of the substance tested, both with and without buffer. The second part is block diagram showing for each point on the graph the changes in pH. Th lower line of the diagram is the initial pH; the upper one gives the pH at th end of the experiment.

RESULTS

The germination inhibiting effects of a number of organic acids related coumarin were examined. The results showing the inhibitory action of thes acids are recorded in Table I. The results show that there is very little relatio between the structure of these acids and their activity. There is nothing indicate that the insertion of a double bond in the side chain with the carbox group is of any significance. This is of particular interest in view of the para mount importance of the double bond in coumarin (Mayer and Evenari It is illustrated by the following examples from Table I: benzoic acid I. 0·25, cinnamic acid 0·17, cinnamyl-acrylic acid 0·29, melilotic acid 0·1 hydrocinnamic acid 0·18.

The results also show clearly that the introduction of an amino group in the nucleus removes activity almost entirely as instanced by anthranilic ar benzoic acids and by *p*-aminosalicylic acid and salicylic acid.

TABLE I

Inhibition indices for germination of lettuce of organic aromatic acids and their salts

2:4-D	5·0	2:4-D Sodium salt	5·0
2:4:5-T	1·1	Potassium *cis*-coumarate	0·5
Indole-acetic acid	0·34	Methyl benzoate	0·36
Salicylic acid	0·33	Methyl cinnamate	0·25
Furylacrylic acid	0·31	Ethyl salicylate	0·25–0·
Cinnamylacrylic acid	0·29	Methyl salicylate	0·2
Benzoic acid	0·25	Ethyl benzoate	0·08
Crotonic acid	0·19	Potassium *trans*-coumarate	0·06
Melilotic acid	0·18	Propyl benzoate	0·04
Hydrocinnamic acid	0·18	Ethyl coumarin-3-carboxylate	0·03
Cinnamic acid	0·17	Butyl benzoate	0·025
Coumarin-3-carboxylic acid	0·15	Potassium salicylate	0·025
Anthranilic acid	0·11	Potassium cinnamate	0·007
Trans-coumaric acid	0·08	Potassium benzoate	0·025
p-Aminosalicylic acid	0·03		
Gallic acid	0·1		

Table I also shows the results regarding the salts and esters of orga acids. The general rule appears to be that the salts of the organic acids a considerably less effective than the corresponding acids. An exception to th is 2:4-D and its salt.

This confirms results obtained for wheat by Ullman (unpublished). The ers of the organic acids show little regularity in their behaviour and may more or less active than the acids themselves. The methyl ester tends be more active and the ethyl one less active than the acid.

Methyl ester > benzoic acid > ethyl ester > propyl ester > butyl ester;
Methyl ester > cinnamic acid.
Salicylic acid > ethyl ester > methyl ester.
Coumarin-3-carboxylic acid > ethyl ester.

An ascent in the homologous series of the esters as instanced by propyl d butyl benzoates does not lead to an increase but rather a further diminu-on of the inhibitory action.

From these results again very little definite emerges regarding the relation tween structure and activity.

A number of previous claims regarding the activity of *cis*- and *trans*-nnamic acids were reinvestigated. The results are shown in Table II. It pears that if there is any real difference between the two optical isomers, e *trans*- and not the *cis*-isomer as previously claimed is the one with the eater inhibitory activity.

TABLE II

Percentage germination of lettuce

olarity × 10^{-3}	1·13		2·26		4·52	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
s-cinnamic acid	77	5	40	22	1	—
ans-cinnamic acid	47	19	18	12	0	—

The claims of Akkerman and Veldstra (1947) regarding the activity of ffeic and ferrulic acids were reinvestigated. From the graphs in Fig. 1 it early emerges that coumarin is incomparatively more active than either of ese two acids, the more active one, ferrulic acid, having an inhibitory index only 0·08. Clearly then neither of these two can be the active principle of mato extracts of Köckemann (1934).

As the question of pH relationships to acid activity arose it was decided to udy the whole problem of the relation between activity and pH as well as vestigating the effect of various buffers on the germination. Ullman (un-blished) records that pH is not the main factor in inhibition and Evenari 949) reached similar conclusions.

It was first attempted to obtain buffers which would permit a study of e various effects. For this purpose two buffers were selected for study: osphate buffers which consisted of potassium dihydrogen phosphate and potassium hydrogen phosphate of various pH, and acetate buffers consisting potassium acetate and acetic acid. In the acetate buffers germination was nly obtained if during the course of the germination appreciable changes in H occurred. This is illustrated by Table III.

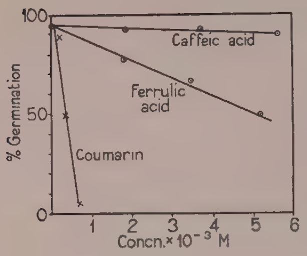

FIG. 1.

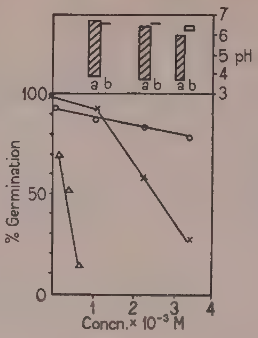

FIG. 2.

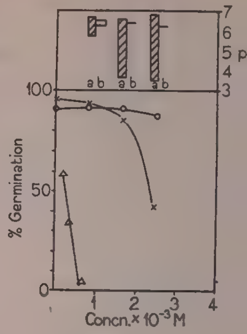

FIG. 3.

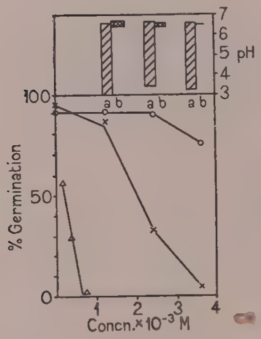

FIG. 4.

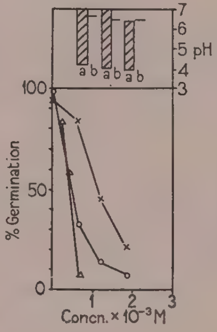

FIG. 5.

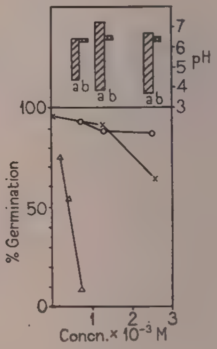

FIG. 6.

FIG. 1. Germination inhibition by caffeic and ferrulic acids.

FIG. 2. Germination inhibition by cinnamic acid (with buffer –○–, without – ×–) and c marin (–△–). Upper portion: *a*, pH without buffer; *b*, with buffer.

FIG. 3. Germination inhibition by benzoic acid (with buffer –○–, without – ×–). Couma and other conventions as in Fig. 2.

FIG. 4. Germination inhibition by salicylic acid. Conventions and coumarin control in Fig. 2.

FIG. 5. Germination inhibition by melilotic acid. Conventions and coumarin control as Fig. 2.

FIG. 6. Germination inhibition by hydrocinnamic acid. Conventions and coumarin contro before.

It is clear from this that in the case of acetate buffer it is neither the ini nor the final pH which is determining but the dilution of the buffer. On other hand, the use of the buffer at dilutions where germination occurs useless as it no longer acts as a buffer. The effect of phosphate buffers is a shown. From this it will be seen that the use of phosphate buffers is legitim from an experimental point of view, as normal germination can be obtai at concentrations where relatively small changes in pH occur during experiment. Equally it is clear that the action of acetate and phosph buffers are different. It was decided in the continuation of the experiments

TABLE III

Germination in acetate and phosphate buffers

	Concentration of buffer	Percentage germination	Increase in pH
Acetate buffer	M/10	0	0·7
pH 3·5	M/40	0	2·7
	M/100	39	3·1
	M/10	0	0·5
pH 5·5	M/40	54	3·3
	M/100	93	2·5
Phosphate buffer	M/10	21·5	0·32
pH 5·2	M/40	94	0·31
	M/100	93	0·44
	M/10	11	0·2
pH 6·5	M/40	94	0·07
	M/100	95	−0·6

use only one buffer, the phosphate buffer at pH 6·5, which was liable to only very slight changes in pH on adding the solutions of the organic acids.

A number of experiments with some of the acids previously studied were conducted. The purpose of this was to compare the germination inhibition obtained when the acid acts alone and when it acts in the presence of a buffer which by itself gives normal germination.

The graphs in figs. 2–6 show the results both of the germination and the pH changes of the external solution (as described under methods). A number of points stand out very clearly from a survey of Figs. 2–6. First of all the external pH of unbuffered solutions of organic acids rises very markedly during the course of the experiment, whereas that of solutions buffered to pH 6·5 remains practically constant. Despite the fact that the pH of the external solution in buffered and unbuffered solutions at the end of the experiment is almost identical, the germination obtained in the two cases is very different. In four of the cases examined the buffered solutions gave much lower inhibitory action than the unbuffered ones. In view of the previously recorded data that salts are less active than the acids, this can be readily understood. In the buffered solutions we are dealing with salts and no longer with the free acids. In the case of melilotic acid, however, the buffered solution is the more active one (Fig. 5). It seems likely that here there is some form of synergism between an active substance and one normally inactive, as has been noted in other cases (Levari, Mayer, and Evenari, 1952). The most important fact is, however, that the germinating seeds act strongly as buffers and can change the pH of the surrounding medium by several pH units.

DISCUSSION

It seems worthwhile to discuss briefly the results obtained in this investigation whose aim was chiefly a completion of a previous paper discussing the relation between structure and activity as germination inhibitors of coumarin and its derivatives. As already pointed out, there does not appear to b any factor or group of factors which determine whether an organic acid i active or inactive as an inhibitor. The acids of the auxin type are really activ only in the case of 2:4-dichlorophenoxyacetic acid. All the other compound of this type were relatively inactive as compared with coumarin. Neither th presence nor absence of a double bond in the side chain nor the number o constituents in the nucleus appear to be governing factors. The nature of th constituents is to some extent of importance in that the introduction of a amino group very effectively reduces activity. Very little evidence could b obtained as to which are the factors which determine activity of an organi acid. The criticism is often levelled at investigations of this nature that th results are invalid unless the pH of the acids and consequently their dissociation constants are taken into account. For this reason the study of p was undertaken. A number of very significant facts emerged. Only a ver limited number of buffers of carefully selected strength may be used i germination studies, due to the effect of the buffer on germination. Whe such a buffer is found further caution is required. It was shown that th activity of acids can be altered in one of two directions by the use of buffer causing either greater or lesser activity. In no case was the activity the same a of the acid alone. Two factors are of importance here. On the one hand, th known fact that the salts of the acids are less active than the acids may caus the decrease in activity observed. On the other hand, synergistic mechanism which is known to occur may cause an increase in activity (Levari, Mayer, and Evenari, 1952). In view of these two possibilities and the fact that the final pH of the buffered and unbuffered solutions is the same, the following general conclusions are inevitable. The use of buffers is neither legitimate nor necessary. It is not legitimate because a number of secondary factors may entirely mask the central issue under investigation. It is unnecessary because the seeds themselves act very powerfully as buffers and are always, even in the case of fairly strong acids, able to bring the external pH to near neutrality. For this reason buffers near neutrality add nothing to the normal functioning of the seeds as buffers, while buffers of low pH act adversely by counteracting the normal buffering action of the seeds.

The only satisfactory method left of taking into account the strength of the acids would therefore be an investigation of their dissociation constants at various concentrations, a study we were unable to undertake within the framework of the work here described.

It is worth pointing out the conclusions regarding the activity of two previously investigated organic acids. It was shown that of the two isomers of cinnamic acid the *cis* is not the more active one. The evidence in fact points to the opposite being the case. In this connexion it is interesting to note that Bonner and Galston (1944) showed *trans*-cinnamic acid to be a natura inhibitor in guayule.

Finally, a study of the inhibitory action of caffeic and ferrulic acids has shown both these acids to be far too weak germination inhibitors in order to e assigned the role of active principal or even part thereof in tomato-juice xtracts as described by Veldstra.

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The Nature of the Resistance of Oats to the Take-all Fungus

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SUMMARY

The two varieties of the take-all fungus, *Ophiobolus graminis* and *O. graminis* var. *Avenae*, show a differential reaction to sap extracted from oats; the type variety, which is incapable of causing a lasting infection of oats *in vivo*, is inhibited by the sap *in vitro*, whereas var. *Avenae*, pathogenic to oats, can grow in the sap. The inhibitory action of the sap is not due to a lack of food material required by the fungus, but to a specific substance or toxin. The method used for assaying toxicity is described.

The toxin is thermostable, moderately stable on storage, virtually insoluble in non-polar solvents, and soluble in acetone, water, and methanol. Methanol extraction of the ether-washed residue of the filtrate from boiled sap from oat leaves results in a semi-purified substance which, when added to 2 per cent. Yeastrel solution, reduces growth of *O. graminis* to half that of the control at a concentration of 10 p.p.m.

The inhibitor is produced in considerable quantity in the leaves and stems of oats and in smaller quantity in the roots. It appears to accumulate mainly during the period of active growth and to be less active, or present in smaller concentration, in adult plants. It can be detected in the seminal roots throughout their existence, although during the first 3 weeks of growth *O. graminis* can invade the cells of the cortex. It is present in greater quantity in the crown roots, which are never penetrated.

A similar inhibitor of *O. graminis* can be extracted from *Arrhenatherum elatius*, but not from other grasses which, both in the field and in pot experiments, appear to be equally resistant.

INTRODUCTION

THE fungus *Ophiobolus graminis* Sacc., causing take-all of wheat, barley, and several grasses, does not cause a lasting infection of oats. Oats are, however, fully susceptible to *O. graminis* var. *Avenae*, a variety first isolated from diseased oats in Wales and differing from the type species in the length of the spores as well as in its wider host range. In an earlier paper (Turner, 1940) the results of an investigation into the resistance of oats to *O. graminis* were described. In the early stages of seedling growth the difference in infection of the seminal roots of oats by *O. graminis* and by *O. graminis* var. *Avenae* is one of degree only, considerable penetration occurring in both cases. After about 3 weeks, however, the cells of the oat roots overcome infection by the type variety and only scattered cells showing persistent hyphae, often enclosed in lignitubers, can be seen. The crown roots, which are produced at about the

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tne that the seminal roots are recovering from infection, show a much more car-cut resistance and may be described as immune; no penetration into the crtical cells has been seen.

The disappearance of the invading hyphae from the seminal roots suggests tat the resistance of oats is of the protoplasmic type (Brown, 1936) or due to a 'plasmatic' defence reaction on the part of the host (Gäumann, 1950). esistance of plants to fungal attack has been traced back in several cases to a constituent of the protoplasm acting in the cell after penetration by the fngus, and in a few cases the active substance has been identified. The most fmiliar example of digestion of invading hyphae occurs in Orchids, where ernard (1911) showed that in orchid tubers the intracellular hyphae of the ycorrhizal fungus are absorbed by the host cell; he prepared an extract fom the tubers which was active for a short period. Burges (1939) confirmed tis work and showed that a thermolabile inhibitory substance exists in the sems, leaves, and tubers of *Orchis incarnata* and is also present, to a much naller extent, in the roots, the only part of the plant usually infected. Other ork in this field has been recently summarized by Gäumann (1950).

A preliminary experiment to test the reaction of the two varieties of *O. aminis* to expressed sap from wheat and oat seedlings indicated the presence a substance in the oat sap toxic to the type variety but not to var. *Avenae.* his substance has been investigated, and it is suggested that its presence in e cells of oat roots confers resistance on the plant.

EXPERIMENTAL RESULTS

Materials. Isolates of *O. graminis* from infected wheat roots (numbers W.6 nd W.7) and an isolate of *O. graminis* var. *Avenae* (No. 0.15) were kindly pplied by Dr. S. D. Garrett. The cereals used were 'Victory' oats, 'Little oss' wheat, and 'Spratt Archer' barley

Production of extract and method of assay. The grain was planted thickly on e surface of sand in wooden flats or flower-pots. These were watered with nutrient solution. After about 14 to 21 days, when the seedlings were some in. high, they were washed free of sand and ground in a mortar or in a Waring lendor with a small quantity of distilled water. Sap was extracted from the oots or from the tops, the latter being cut off just above the seed. After rinding, the juice was expressed and, in most cases, boiled to inactivate nzymes. On boiling, a slight precipitate was produced in the root extract nd a heavy green coagulum in the extract from the tops. The juice was then ested, either directly or after further treatment, for its effect on growth of the ungal isolates.

In the preliminary experiment autoclaved but otherwise untreated juices rom wheat and oat roots were put into 50 ml. conical flasks, 2 ml. per flask, nd the flasks were plugged with cotton-wool and autoclaved at 10 lb. pressure or 10 minutes. They were then inoculated with isolates W.6 and O.15; the noculum was in all cases a disk, 7 mm. in diameter, cut with a cork borer from he growing edge of a colony on potato dextrose agar and transferred with a needle to the centre of the flask. Four flasks were inoculated in each of t four series. Growth was recorded by measuring the diameter of the fung mat on the bottom of the flask. Both isolates grew rapidly on the wheat juic reaching a diameter of 4·2 to 4·5 cm. (the size of the base of the flasks) in days. In the oat juice there was no growth of either isolate for 4 days; the c isolate then grew slowly and covered the bottom of the flasks in 8 to 10 day but the wheat isolate showed no growth and the inoculum disintegrated. T pH in all flasks after autoclaving was between 5·5 and 6·0. Where grow occurred, the final pH was between 7·0 and 8·0.

Validity of assay method. The technique described above was adopted the standard method of assaying activity of extracts or fractions of extrac Possible objections based on the effect of autoclaving on the juices, and t source of the extract, were taken into consideration.

Two methods were used to test the possible effect of autoclaving on inhib tory action by the oat juice.

i. Part of the method used by Burges (1939) in his investigations of t effects of extracted cell contents of orchids on the mycorrhizal endophy was followed. Agar smears were prepared by painting sterile coverslips wi quarter-strength potato-glucose agar. These were inoculated at one side wi the fungus, inverted over ring cells, and incubated in damp chambers. Wh the hyphae had grown half-way across the coverslip, the material to be treat was placed on the agar ahead of them and the reaction of the hyphae w noted at intervals.

Extracts of wheat and oat roots were treated and tested in the followi forms: (*a*) raw extracts; (*b*) extracts boiled and centrifuged, and sediment a supernatant liquor tested separately; (*c*) extracts filtered through a Sei filter. The results are summarized in Table I.

TABLE I

Effect of extracts of oat roots on growth of W.6 and O.15 across agar-smear coverslips

0 = growth stopped and hyphae killed; 1 = growth checked; 2 = growth norma

	Untreated		Boiled and centrifuged				Filtered	
			Sediment		Supernatant			
Isolate	W.6	O.15	W.6	O.15	W.6	O.15	W.6	O.15
Oat extract	0	1	0	1	1	2	2	2
Wheat extract	2	2	2	2	2	2	2	2

After 24 hours the raw oat juice and the sediment obtained after centrifugi this juice had caused the death of the hyphae of the wheat isolate; the branched repeatedly just behind the tip, became vacuolated, and final disintegrated (see Fig. 1). The supernatant liquor from the same juice check growth but caused little disintegration, and in some cases growth was resum 24 hours later. Seitz-filtered juice had no toxic effect whatever, neither did a wheat extracts. The oat isolates reacted much less strongly to the active o extracts.

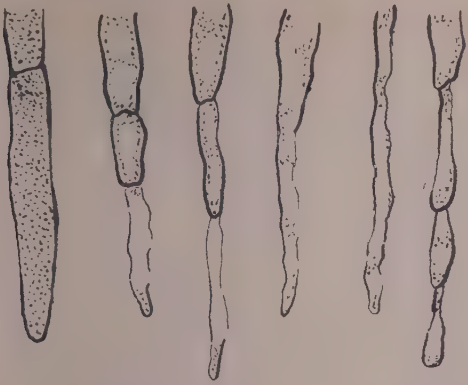

FIG. 1. Examples of hyphae of isolate W.6 disintegrating under the action of oat juice. A healthy hypha is shown on the left.

ii. The liquids were sterilized by passage through either a Seitz filter or hrough a sintered glass filter of maximum pore size, 1·8μ. The filtrate was oured into 50-ml. flasks, half of which were subsequently autoclaved. All ere then inoculated with isolate W.6. Results are shown in Table II. A iameter of 0·7 cm. means that no growth has occurred, since this is the size the inoculum.

TABLE II

verage colony diameter in cm. of W.6 in filtered and in autoclaved juices from wheat and oats respectively

	Filtered juice (Seitz)				Filtered juice (sintered glass)					
Age in	Not autoclaved		Autoclaved		Not autoclaved		Autoclaved		Autoclaved juice	
ays	Oats	Wheat	Oats	Wheat	Oats	Wheat	Oats	Wheat	Oats	Wheat
1	1·2	1·0	1·3	1·5	0·7	1·0	0·8	1·3	0·7	1·7
3	2·8	2·7	3·2	3·0	0·9	2·8	0·8	3·0	1·1	3·8
6	4+	4+	4+	4+	1·0	4+	0·9	4+	1·6	4+

he oat juice filtered through a Seitz paper was thus not toxic to the wheat solate, and subsequent autoclaving had no effect. When filtered through intered glass the oat juice was toxic whether subsequently autoclaved or not. he unfiltered autoclaved juice was less toxic here than in the preliminary xperiment, and rather less toxic than the juice filtered through sintered glass. All the filtered juices were clear, colourless liquids and were probably low in nutrient content. The much greater removal of the toxin by the Seitz filter s assumed to be due to its greater adsorptive power. Centrifuging the boiled oot extract produced much the same effect as did the sintered glass; both the ediment and the supernatant liquor were toxic.

These results, taken together, show that the toxic effect of the extract fro oat roots is a property of the raw extract and is not produced on autoclavin They also suggest that the toxin is present in low concentration in extrac from the roots and is removed by adsorption on to a Seitz filter-pad; it is al partly adsorbed on to the precipitate formed on boiling.

The part of the plant extracted and the age of the plant were found to ha a considerable effect on the degree of toxicity of the extract towards t wheat isolates. When juice was extracted from the tops of oat seedlin instead of from the roots, a higher level of activity was obtained. A hea green coagulum was formed on boiling. After centrifuging, the supernata liquid was toxic, but the greatest activity was in the sediment. When t supernatant liquor was passed through a Seitz filter the filtrate was st slightly active, suggesting that the adsorptive capacity of the filter-pad ha been saturated. The highest yield of toxin can thus be obtained from extrac of seedling tops and in these extracts the greatest activity resides in t coagulum. In later attempts to get the active material in a purer state, jui from the tops was boiled and filtered through a Buchner funnel, the filtra was discarded, and the toxin removed from the residue by various solvent In this way much of the sugars and other water-soluble substances we eliminated.

A comparison between equal volumes of extract from tops of adult plan and of seedlings shows a slightly diminished activity on the part of the forme but some activity is present throughout the life of the plant. Sap from plan more than 5 weeks old yields a less flocculent precipitate on boiling, and whe the boiled juice is filtered, a larger proportion of the toxin is to be found i the filtrate and less in the residue.

Extracts from different parts of seedlings 2 to 4 weeks old were compare and the results indicated that the toxin accumulated chiefly in the leaves an young parts of the plant, and that a diminishing concentration-gradie exists from the top of the shoot to the roots. Sap from the crown roots showe greater toxic activity than did sap from the seminal roots; the former we growing vigorously at the time the extracts were made, whereas the latt had stopped active growth.

Differential isolation of the toxin in various solvents. Residues after filtrati of juice from oat tops were extracted with different solvents. The extrac were evaporated to dryness in a 60° C. oven, weighed, and taken up again i the same solvent. The concentration was adjusted on a weight volume bas and the solution was tested at various concentrations. Two ml. of the liqui at each dilution were pipetted into each of four 50-ml. flasks. With all volati solvents, the solvent was driven off in a 60° C. oven and replaced by 2 per cen Yeastrel. The dilute acid and alkaline solvents were neutralized, and these an the distilled water series were also brought up to a concentration of 2 p cent. Yeastrel. To test the efficiency of water as a solvent, the residue w first extracted with methanol. This removed the material adsorbed on to t coagulum and, after drying the resulting solution, the residue was taken up i stilled water. A blank was run with each solvent and no solvent toxicity wards the fungus was found. The results are given in Fig. 2.

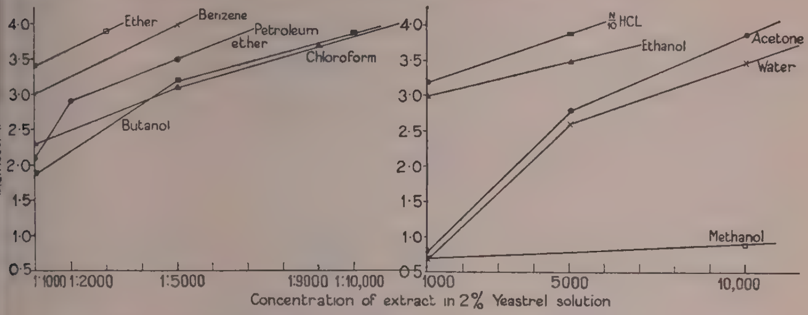

FIG. 2. Fungal growth in material extracted by various solvents.

In Table III are given the minimum concentrations of the fractions taken p in the various solvents which reduce growth after 3 days by 50 per cent. f the control.

TABLE III

Growth of W.6 in fractions of oat juice taken up in different solvents and tested in per cent. Yeastrel. Minimal concentrations giving 50 per cent. growth reduction are shown

Benzene	More than	1:1,000	Petrol ether	1:1,000
Chloroform	,,	,,	0·1 N.Na_2CO_3	1:2,500
Ether	,,	,,	Acetone	1:3,000
Ethanol	,,	,,	Water	1:3,000
·1 N.HCl	,,	,,	Methanol	1:15,000
Butanol	1:1,000		Water after ether wash	1:15,000
			Methanol after ether wash	1:100,000

Methanol is thus the most efficient solvent and if extraction with methanol is preceded by washing with ether, a high level of activity is obtained.

Stability of the toxin. A methanol extract of the ether-washed residue of iltration of boiled oat-leaf juice was dried in a 40° C. oven, stored in a desiccator, and at intervals taken up again in methanol and tested for toxicity to *O. graminis*. Results given in Table IV show a loss of activity in the first 20 days. There occurs also a rather greater reduction in solubility in methanol of the material. Some process of denaturing is therefore occurring, but the substance is still impure and whether it is the toxin or associated impurities which alter in solubility has not been determined.

Occurrence of similar inhibitors in other members of the Gramineae. Extracts of leaves of barley and of various grasses were filtered and the residues extracted with methanol. The methanol-soluble fraction was tested at a concentration of 1:1,000 in 2 per cent. Yeastrel for toxicity to fungus isolate W.7. Results are given in Table V.

In a previous paper (Turner, 1940) investigations into the susceptibility of barley and various grasses were referred to. Barley was found to be as

TABLE IV

Growth of W.7 in 2 per cent. Yeastrel showing the effect of storage in a desiccator on two samples of the toxin. Diameters in cm.

Concentration of toxin	1:50,000		1:100,000		Length of storage period, in days
Age of colony in days:	3	4	3	4	
Sample 1	1·6	1·8	3·1	3·5	0
	2·3	2·9	3·7	4+	20
	2·1	3·0	4+	—	90
Sample 2	0·8	1·0	2·3	2·9	0
	2·2	2·9	3·8	4+	35
	2·3	2·9	4+	—	60

TABLE V

Growth of W.7 in methanol-soluble fraction of sap residue of barley and various grasses

Plant	Diameter in cm.	Plant	Diameter in cm.
Control	3·6	*Anthoxanthum odoratum*	3·3
Barley	3·1	*Dactylis glomerata*	3·4
Agrostis vulgaris	3·9	*Lolium italicum*	3·5
Arrhenatherum elatius	0·7	*L. perenne*	3·4
Trisetum flavescens	3·6	*Phleum pratense*	3·4

susceptible as wheat to the different isolates used, and there was no significant difference in degree of attack of either wheat or barley by the wheat and oat isolates. Results of pathogenicity tests for the above grasses (given in Table VI) showed considerable variation in pathogenicity between different isolates of the same variety of fungus.

TABLE VI

Extent of discoloration of vascular strand of seminal roots of grass species inoculated with fungus isolates from wheat and oats; the results are expressed as a percentage of the total length of root

	Isolate					
Grass species	W.1	W.2	W.3	O.1	O.4	O.13
Agrostis vulgaris	1	3	53	76	57	—
Anthoxanthum odoratum	11	2	19	68	67	16
Arrhenatherum elatius	5	2	6	59	49	16
Trisetum flavescens	25	6	3	89	81	50
Dactylis glomerata	9	7	21	73	49	32
Lolium italicum	60	10	87	64	60	53
L. perenne	62	38	51	78	78	52
Phleum pratense	18	10	42	63	50	64

These results showed that infection by the O. isolates was in all cases more severe than that by the W. isolates and that there was no case of complete resistance of a grass to all isolates. *A. elatius* showed the most sustained resistance to the wheat isolates; *T. flavescens*, *Anthoxanthum odoratum*, and *Dactylis glomerata* were slightly susceptible, the two *Lolium* species very susceptible, and *Agrostis vulgaris* and *Phleum pratense* intermediate, but dfering widely in their reaction to the three W. isolates.

Garrett (1940) summarizes previous work on relative susceptibility of dferent grass species to infection by *O. graminis* and confirms by results of h own experiments the susceptibility of species of *Bromus*, *Dactylis*, *Lolium* &., and the relative resistance of species of *Avena* and of *Phleum pratense*.

Thus, although clear-cut answers of susceptibility or resistance to *O. graminis* are difficult to obtain with grasses, it is clear that *Anthoxanthum odoratum*, *A. elatius*, *T. flavescens*, and *Phleum pratense* show comparable dgrees of resistance to infection. *Arrhenatherum elatius* is the only one of the grasses so far tested which produces a substance inhibiting growth of *O. graminis in vitro*, suggesting that resistance to infection in this case is cmparable in type to that of oats. Resistance in the other three grasses is therefore presumed to be due to other causes.

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A Germination Inhibitor from Sugar-Beet

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SUMMARY

There is present in the water extract of sugar-beet seed balls an unsaturated yellow oil which is capable of inhibiting germination of various seeds. It also inhibits salt uptake and the respiration of sugar-beet tissue disks. The activity of the polyphenolase enzyme present in beet tissue is also depressed. The role of the oil in inhibition of sugar-beet germination is discussed.

A RECENT comprehensive review of our knowledge of germination inhibit (Evenari, 1949) obviates any need for a preliminary survey of the literatu in this article.

That sugar-beet seeds do not readily germinate is well known to grow and seedsmen. The actual cause of the delayed germination has been variou attributed. Henley and Woodman (1930) found that treatment with dilu sulphuric acid, which in their opinion increased the permeability of the see coat, increased the rate of germination. According to Froeschel (1940) a Tolman and Stout (1940) the seed-coat contains substances which inhibit t germination of sugar-beet and other seeds. Stout and Tolman (1941) clai that, when beet seeds germinate, free ammonia is liberated from nitrogeno substances present in the seed-coat. They consider that the ammonia, libe ated by a hydrolytic enzyme, is produced in quantities sufficient to exert toxic action on the growth of the young embryo. Ammonia liberation in pla tissue can only be caused by a limited number of enzymes: it may, for instanc occur through the medium of an *ortho*-quinone produced by a polyphenola acting on free amino-acids (James, Roberts, Beevers, and de Kock, 194 Stout and Tolman (1941) themselves suggested urease and asparaginase, b could only find slight indications of urease. It is questionable that su enzymes exist in the dead tissue forming the pericarp round the true see and yet to support the view that ammonia is liberated from the seed-coat enzyme activity the enzymes would have to occur *in situ* as it is unlikely th they would be secreted from the embryo.

Duym, Komen, Ultée, and van der Weide (1947) have shown that t osmotic effects of substances contained in the seed ball are important relation to germination, but they failed to demonstrate a specific inhibito substance.

In a previous note (de Kock and Hunter, 1950) we reported the separati from the water extract of sugar-beet seeds of an unsaturated yellow oil whi inhibits the germination of both sugar-beet and other seeds and has a mark

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ffect on root growth. Subsequent to this communication, Rees and Skelding 1950) claim to have demonstrated in the tissue of red beet an unidentified hibitor which prevents salt absorption and is removed on prolonged washing.

MATERIALS AND METHODS

The sugar-beet (*Beta vulgaris* L.) seed balls used in these studies were btained from five different seedsmen, two of them local. No attempt was ade to secure seed of one variety only, but significant differences were not found between different samples.

Water extracts were made simply by steeping the seeds in sufficient glass istilled water to cover them completely, leaving them overnight at room emperature, and filtering off the liquid through a Buchner funnel. This xtract, containing the water-soluble substances from the beet seeds, was then mployed in germination tests.

In attempting to find an ether-soluble inhibitor in the water extract the xtract was acidified with dilute sulphuric acid and repeatedly treated with peroxide-free ether. The ether used was Analar peroxide-free ether, thrice distilled through an all-glass apparatus, as it became evident that commercial ether contained substances which would inhibit germination and ether itself would extract inhibiting substances from rubber bungs. As a check therefore the last 3 ml. of the ether from the final distillation were allowed to evaporate from a small filter-paper which was then placed in the inner ring of a Conway Unit moistened with 1 ml. water and containing 25 cress (*Lepidium sativum*) seeds set out to germinate on it in the dark at 16·5°. If germination agreed with a control containing distilled water only, the ether was deemed suitable.

Extraction was carried out in 1·5-l. separating funnels, using 600-ml. portions of water extract and three successive 50-ml. portions of ether. The ether fractions were bulked, allowed to stand overnight till no more water separated, dried with anhydrous sodium sulphate, and the ether distilled off. The resulting sweet-smelling oil was freed from ether by blowing over it a slow current of air. The oils extracted from different samples varied in colour from pale yellow to brown and were more viscous in some batches. On prolonged standing the odour became somewhat rancid and the colour paler. This affects the potency, the lighter being more potent, and the fresher the seed the more potent the oil yielded.

In the germination experiments the test solution used was either the water extract or a solution of the oil at varying concentrations. The oil solution was similarly used in the respiration and salt absorption experiments. Germination tests were carried out in Petri dishes into which a circle of Whatman's No. 1 filter-paper was introduced. Six ml. of the test solution were added after 50 or 100 seeds had been set out on the filter-paper. The Petri dishes were kept in the dark in a constant temperature room at 16·5°. To obtain beet seeds free from inhibiting substances, a number of seeds were secured in a muslin bag under a tap and water allowed to run over them for 12 hours. They were then dried over slight heat and left in a constant temperature room for 3 days until they had attained constant weight. These were then used in germinati experiments as controls.

Respiration and salt absorption experiments were carried out with disks tissue cut from the beet. In early experiments disks were obtained by striki cylinders of tissue from a mature sugar-beet with a cork borer of 1 c diameter and sectioning these into disks of 1 mm. thickness, using a sha razor. In later experiments on salt absorption in which large numbers of dis were required a sugar-beet was sliced by means of a bacon slicer and dis were cut from 5 to 6 slices at a time, using a sharp cork borer. It has been su gested by Turner (1938) that slicing the roots first with subsequent cutti of disks from the slices minimizes the bruising of the tissue.

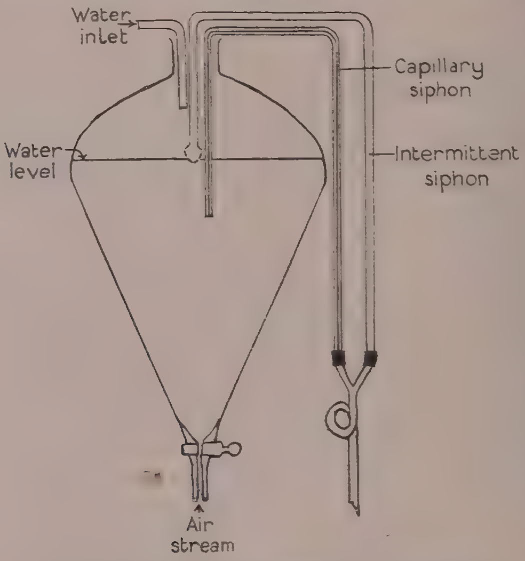

FIG. 1. Reservoir for maintenance of tissue disks.

Prior to use, the disks were kept in a reservoir of aerated tap-water (Fig. The reservoir was a 1·5-l. separating flask into which tap-water was co tinually running, a constant level being maintained by the operation of intermittent siphon. The siphon consisted of a Y-piece, the descendi limb of which was attached to a 20-cm. long glass tube bent through 360° at upper end. To one arm was attached a capillary siphon and to the othe siphon of wide glass tubing, the end of which determined the level of water the container. The capillary siphon would operate automatically and would th fill the 360° bend, which would itself siphon over and so provide sufficient p to set off the wide glass siphon. This siphon arrangement operated over extend periods even when a strong stream of air was blown into the container.

The water was aerated by a current of air supplied by a Proctor Hea Duty air pump. By permitting the air supply to enter the reservoir throu he stem of the flask the air current served the dual purpose of aerating the water and of keeping the disks in constant circulation entirely free from one nother, thus preventing the development of anaerobic conditions. It also erved to ensure that no bacteria could long remain on the disks. In this pparatus it is possible to keep up to 1,200 disks in a condition which will permit their being used for investigations such as those described in this paper rom 1 to 600 hours.

Respiration rates were determined by standard Warburg techniques. In hese estimations ten disks of uniform size and in a firm healthy condition were used per vessel and after being carefully blotted dry were immersed in a olution of volume 2·5 ml. contained in the annulus, there being 0·2 ml. per cent. KOH absorbed on a fluted filter-paper in the centre cup. The ubstance, the effect of which was under investigation, was added to the side rm and, at zero time, was tipped into the main compartment of the vessel. The vessels were immersed in a water-bath kept at 20° by a sensitive thermotat. Readings were taken every 30 minutes for a period of 2 hours after quilibration. Respiration readings are expressed as μl. oxygen absorbed per 00 mg. dry weight.

The experimental fluid used in the phosphate uptake experiments was a uitably diluted Sorensen's phosphate buffer solution of pH 6·5, a pH which approximated to the pH of the tissue. The phosphate estimations were usually carried out every 30 minutes on the solution from the Warburg vessels in which the disks were respiring by adding 1 ml. ammonium molybdate to 1 ml. test solution followed by 1 ml. hydrazine hydrochloride, warming after suitable dilution to 100°, and estimating the colour intensity on a 'Spekker' photo-electric absorptiometer. This method of following phosphate absorption has the advantage that the ratio of tissue to solution is always constant: 10 disks to 2·5 ml. solution.

To identify amino-acids present in the seed-coat one-dimensional paper chromatograms were run on a concentrated sugar-beet seed extract in the usual manner (Consden, Gordon, and Martin, 1944), using phenol-water as solvent. Later two-dimensional chromatograms with butanol-pyridine-water as second solvent were employed. De-salting was carried out electrically or by using pyridine.

EXPERIMENTAL RESULTS

(a) *Germination*

Germination tests using sugar-beet in the presence of other seeds showed conclusively that the beet seeds have an inhibiting action on the germination and growth of sugar-beet and other seeds. As this has been demonstrated by a number of earlier investigators (Froeschel, 1940; Stout and Tolman, 1941), further results are not presented here.

It was apparent, however, when using cress seeds that the growth of the radicle was much inhibited while the hypocotyls were only slightly affected. This is shown by the data given in Table I, based upon an experiment in which beet seeds were placed at the centre of the Petri dish and cress see arranged around the perimeter. The root-tips of the cress seedlings we characteristically blackened, as were those of the beet seeds. This fact w observed by Stout and Tolman (1941) and attributed to the liberation ammonia by enzymes present in the seed-coat acting on nitrogenous sul stances.

TABLE I

Cress-seedling measurements (inches × 10^{-1}) when placed round perimeter of Pet dishes with beet-seed clusters in centre. Means and standard errors of 25 seedling

Seed clusters per dish	Inner ring of seedlings		Outer ring of seedling	
	Radicle length	Hypocotyl length	Radicle length	Hypocoty length
5	11·54±0·95	8·96±0·68	17·48±1·13	10·04±0·5
10	7·09±0·63	6·83±0·60	11·79±0·79	9·38±0·6
0 (control)	18·9±1·01	7·6±0·3		

Experiments using the water extract from beet seeds described earli showed that it was as effective as the beet seeds themselves in preventing t germination of cress and other seeds. Seeds of cress, beet, and Italian r grass (*Lolium italicum*) were germinated in Petri dishes in the dark at 16· using the water extract above and also dilutions of half and two-thirds, usi 6 ml. per Petri dish. Controls had glass distilled water. The germination w noted daily for 11 days and is presented in Table II.

TABLE II

Percentage germination of Lepidium sativum, Lolium italicum, and Beta sacchar fera in water extract of beet-seeds at three dilutions

	Concentration of extract	Days from sowing					
		1	3	5	7	9	11
Lepidium	Full strength	0	0	0	2	3	3
	0·5 ,,	0	2	8	15	15	19
	0·33 ,,	0	13	15	16	68	72
	Control	0	98	98	98	98	98
Lolium	Full strength	0	0	2	13	20	22
	0·5 ,,	0	0	36	54	60	63
	0·33 ,,	0	23	63	70	72	77
	Control	0	23	54	65	70	77
Beta	Full strength	0	0	4	—	—	—
	0·5 ,,	0	0	15	—	—	—
	0·33 ,,	0	7	51	—	—	—
	Control	0	34	72	—	—	—

If cress seeds which have been inhibited by the sugar-beet seed wat extract are washed and set out for germination, the rate at which they germina is remarkably rapid and the hypocotyls are much stouter than the controls. can be concluded from this that some part of the growth process is bei retarded whereas other processes may proceed normally or be stimulated

To test the effect of the oil on germination of cress seeds 50 seeds were set ut in a Conway unit and water containing oil at various concentrations was lded. Table III shows the germination figures obtained and gives germinaon percentages and weights of radicle and hypocotyl. It is apparent that oth the oil and the original water extract markedly affect the length of the dicle.

TABLE III

resh weights (g.) per 100 seeds of hypocotyls and radicles after 6 days germination in water with added ether extract

Ether extract (p.p.m.)	Hypocotyl	Radicle	Total wt.	Ratio radicle/ hypocotyl	% Germination after number days shown in brackets
1,000	—	—	—	—	23 (4)
100	0·396	0·126	0·522	0·31	82 (6)
50	0·619	0·170	0·789	0·26	90 (6)
10	0·735	0·277	1·012	0·37	100 (6)
Control	0·793	0·391	1·184	0·49	100 (4)
					100 (6)

(*b*) *Respiration.* In earlier respiration experiments 14 disks suspended in H 5·5 Sorensen's phosphate buffer were used per Warburg vessel. The hibitor oil was weighed directly into each vessel to give 1 part per 1,000 nal concentration. Fig. 2 gives the result of such an experiment.

In order to determine whether the inhibitor affected the rate of oxidation f catechol by the polyphenolase of sugar-beet, 100 disks were ground with and in 10 ml. water, the extract filtered through muslin, centrifuged for minutes at 3,000 r.p.m., and used as a source of the enzyme. Of this 0·5 ml. vas added to each vessel together with 0·5 ml. pH6 phosphate buffer and ·5 ml. 0·05 M. catechol tipped in from the side arm. The oil was added to give a final concentration of 1 part per 250. The results obtained are presented n Fig. 3. Confirmation of the findings of the previous experiment was obained by investigating the effect of the oil inhibitor on the oxidation of atechol by the polyphenolase of the mealworm (*Tenebrio mollitor*). Mealvorms (2·13 g.) were ground in 30 ml. pH 6 phosphate buffer and then iltered through a Whatman's No. 1. The purplish clear liquid was used in Warburg vessels as above with 0·5 ml. 0·05 M. catechol added from the side arm and oil weighed in directly to give 1 part per 1,000 final concentration. The magnitude of the inhibition obtained is also shown in Fig. 3.

(*c*) *Phosphate absorption.* Experiments on the uptake of phosphate by sugar-beet disks in the presence of the oil were conducted as outlined in an earlier section. At the end of a 3-hour experiment in which 10 disks were allowed to respire in 2·5 ml. phosphate solution contained in a Warburg vessel, the percentage uptake of phosphate was determined; four controls had absorbed 85·0, 80·0, 83·5, and 81·4 and four series in presence of oil absorbed 30·7, 28·5, 32·1, and 30·7. No exosmosis of phosphate occurre when disks were left in distilled water in presence of the inhibitor oil.

In a similar experiment phosphate determinations were made every 3

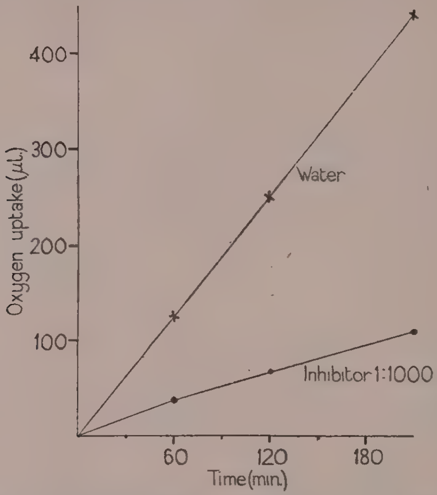

FIG. 2. Respiration of sugar-beet disks in water and with inhibitor (1 in 1,000).

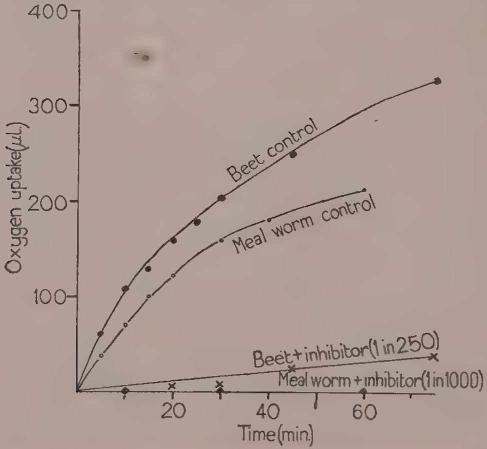

FIG. 3. Oxygen uptake by beet and mealworm enzyme preparations in presence of inhibi and controls in water.

minutes. The oil was added to give a final concentration of 1 part per 1,0 Respiration readings were taken over a period of 2·5 hours (Fig. 4). previously, the disks, in the absence of inhibitor, absorbed nearly 50 per ce more phosphate than did inhibited disks. It is quite evident that the oil ha strong inhibitory effect on the absorption of phosphate by sugar-beet tiss

(*d*) *Biochemical investigations.* To investigate the possibility of ammo beration, urease experiments were carried out by soaking 50 g. seed in 50 ml. glass distilled water. The liquid was filtered off and 20 ml. urea 0·02 M.) added to a 20-ml. aliquot. Controls had no urea added. Ammonia stimations were made after 4 hours using the Conway technique (Conway,

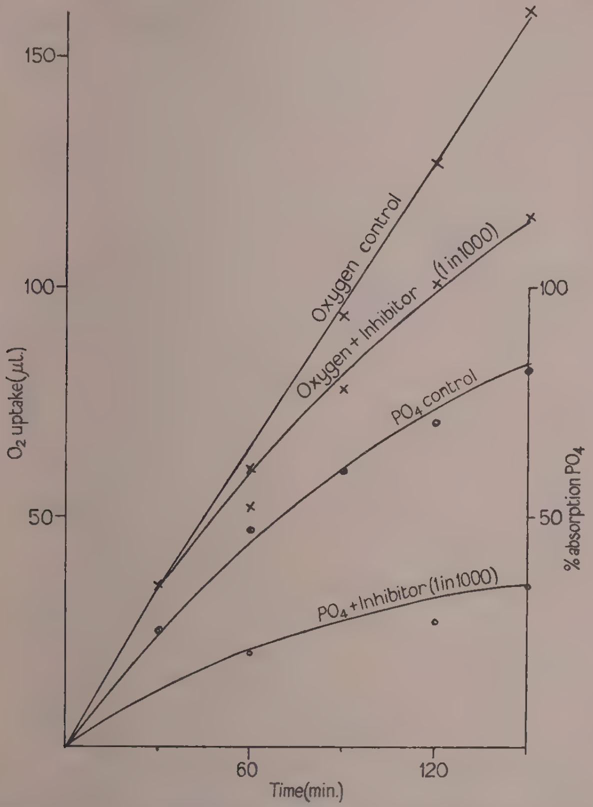

FIG. 4. Oxygen and phosphate uptake by disks of sugar-beet in presence and absence of inhibitor.

1947). Ammonia produced in excess of controls was small. Even if seeds soaked in a phosphate-citrate buffer were used, very little ammonia liberation resulted. Similar experiments were performed with aspartic and glutamic acids over a 4-hour period, using a pH 7·8 phosphate buffer. No increase in free ammonia could be detected, a finding suggesting the absence of the appropriate enzymes.

Moreover, using Warburg manometers, no catechol oxidising enzyme was found in the water extract of sugar-beet seeds. Sugar-beet seeds ground in a hand-mill likewise showed no polyphenolase, although mature beet tissue contains this enzyme.

An analysis of the water extract for amino-acids indicated the presence of leucine +++++; alanine, valine and glycine ++++; γ-amino-*n*-butyric and aspartic acids +++; glutamic acid, serine, and threonine +. The comparatively high proportion of glycine is probably significant in relation to biological inhibition.

Sugar-beet seeds store starch although starch is not found in the root. A water extract of the seed balls contains amylase in quantity. Investigations have so far failed to reveal the presence of other enzymes. The ether extractable oil from the beet seeds has a marked stimulating effect on the amylase as measured by the hydrolysis of soluble starch (Somogyi, 1937). It is hoped to present these experimental results in due course.

(*e*) *Chemical investigations.* Little information on the chemical nature of the oil inhibitor has so far been obtained, due to the low yield of oil (0·2 g. per kg.). No characteristic colour is given with aqueous ferric chloride. On treatment with hydrogen peroxide the inhibitory activity is only slightly lowered Unsaturation is indicated by the fact that iodine water is decolorized by the oil and it also goes rancid on standing. It is quite possible that the oil is a mixture of substances which may act synergistically.

DISCUSSION

Biological inhibitors are widespread in nature. Of these, bacterial inhibitors or antibiotics have received special prominence in recent years. Growth-inhibiting substances are, however, common among higher plants (Peyronel 1947). Koeckemann (1934) was one of the first to draw attention to the existence of specific substances, the effect of which was to delay germination He cited the case of tomato seeds which fail to germinate although present in an apparently ideal medium and suggested that this was due to the presence of inhibiting substances which he named blastocholine. These have been subsequently identified as a mixture of caffeic and ferulic acids which act synergistically (Akkerman and Veldstra, 1947). The seeds of *Melilotus officinalis* similarly fail to germinate because they contain coumarin in quantity (Zwenger and Bodenbrender, 1863). Others (Walger, Markus, and Nagymihaly, 1949) have shown that pumpkin (*Cucurbita maxima*) seeds are inhibited by the presence of volatile substances in the pumpkin.

Osmotic effects can also result in inhibition of germination. A high salinity prevents the germination of cress; the colloidal covering of the seed-coat fails to hydrate and the critical osmotic value of the embryo is probably exceeded However, sugar-beet is a typical halophyte and is physiologically adapted to soils of high salinity. In fact yield of sugar is improved by adding common salt to agricultural soil. It is possible therefore that it is less seriously affected by high osmotic values.

The full significance of this inhibition of germination is not evident in the plant's economy. A possible ecological significance of the inhibiting substances present in sugar-beet has been demonstrated by Froeschel and Funke (1941) They noted the absence of the weed *Agrostemma Githago* from beet fields and showed that when *Agrostemma* and beet were sown together, the *Agrostemma* failed to germinate. The inhibitors may thus have a significance in relation to competition. The presence of a water-soluble germination inhibitor in the seed may be advantageous in that it would prevent germination and possible desiccation until the soil has attained a sufficient moisture content, by which time the inhibitor would be washed out. However, it may be the function of the inhibitor to maintain the seed only partially dormant. For instance, respiration may be suppressed while food reserves are made available by hydrolysing enzymes which continue to function, so that, when the inhibitor is finally removed, abundant energy is available for rapid growth. Evidence has been presented to show that in the presence of the extracted oil, respiration of the tissue is effectively inhibited, while amylase activity continues unchecked or at increased speed. The controlling mechanism is more likely to be endogenous and the result of a specific inhibitory substance. In this connexion it may be noted that Dufrénoy and Pratt (1948) have obtained similar responses with dithiobiuret on rice (*Oryza sativa*) seedlings. Stunting of the roots was obtained at 10 p.p.m. dithiobiuret, but elongation rapidly ensued on transfer to pure water. They suggested that the dithiobiuret was functioning by protecting phenolic substances from oxidation.

It is now generally agreed that there is a close relation between respiration and salt absorption (Robertson, 1950). From our results it will be evident that the inhibitor oil prevents respiration and salt uptake equally effectively. Rees and Skelding (1950) reported the presence in the storage tissue of red beet of a water-soluble substance which inhibited the absorption of salt by disks of beet tissue. That a similar substance occurs in sugar-beet is certainly feasible. In the preliminary work of the present investigation it became clear that the disks had to be washed in the storage apparatus for 4–5 days before it was possible to record an appreciable uptake of phosphate under our experimental conditions. This could suggest the presence in the tissues of an inhibiting substance which is removed on prolonged washing.

Our results would further seem to suggest that ammonia must occupy a minor role in any evaluation of the factors causing inhibition of germination in sugar-beet. It is at least questionable whether ammonia would be produced in quantity sufficient to cause inhibition. About 3·8 m.eq./NH_3/100 ml. water extract has been recorded (Duym *et al.*, 1947), and it has not been possible to demonstrate the activity of enzymes able to liberate ammonia from nitrogenous substances in the seed-coat. The possibility is rendered less likely in acid soils, where the ammonia would be rapidly absorbed by the soil colloids. Of the nitrogenous substances present in the seed coat, glycine may exert some inhibitory activity, as has been shown by Audus and Quastel (1947).

That osmotic effects must have a considerable influence on the embryo is not to be denied. Other factors such as the presence of glycine or free ammonia may also make small contributions to the delayed germination which is characteristic of sugar-beet seeds. But the fact that we have been able to isolate from the seed balls an unsaturated oil which can be shown to cause a striking inhibition of seed germination and of respiration and salt uptake of tissues would seem to suggest that the oil must play a decided role in t plant's metabolism and germination behaviour.

ACKNOWLEDGEMENTS

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On a New Method for measuring the Stomatal Aperture of Leaves

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SUMMARY

A method is described of following the behaviour of stomata by utilizing a diffusion phenomenon, the Dufour effect. Hydrogen at laboratory temperature is blown on to one side of the leaf. As it diffuses through the leaf and out the other side it is accompanied by a rapid temperature wave of considerable magnitude. This is picked up by a fine thermocouple near the leaf and passed to a quick period galvanometer. The 'flick' of this measures the stomatal conductance.

An experiment is described in which the behaviour of a wheat leaf was followed. The results agree well in broad outline with those obtained by other methods.

Advantages claimed are that an approximation, at least, is obtained to the value of the important *diffusive* conductance of the stomata; that the leaf does not have to be enclosed in any way, and that the instrument can easily be made automatic and recording. Ambient temperature changes are not troublesome.

THE purpose of this paper is to describe a new method for investigating the stomatal behaviour of leaves. The principle is, so far as is known, quite new and it has the advantage of depending on a diffusion phenomenon. To some extent therefore it gives information directly applicable to an analysis of the way in which stomata actually control the processes of carbon-dioxide supply and water loss. Before describing the method itself it will be helpful to make a few remarks on the physical principles underlying it.

The Dufour effect. It is a phenomenon found very widely in the physical world in which two agencies of quite distinct nature often interfere with each other to produce additive results in the spheres of each. About 1872, Dufour discovered that when two gases at the same temperature are allowed to diffuse into one another temperature differences develop over the region of inter-diffusion. Then independently in 1917 Chapman in England and Enskog in Sweden, investigating gases mathematically by the kinetic theory, predicted the converse; i.e. that if a mixture of gases was placed in a non-uniform temperature field there would be a partial separation of the components. Dootson confirmed this experimentally the same year. As a matter of fact Soret had found this result for liquids as early as 1893, but it was the mathematical analysis of Chapman and Enskog which revealed the interesting fact that the effect depended on the way the molecules of the gases repelled one another when 'deformed' on impact. The repulsive force between two molecules in 'contact' can be written in a simplified way as Gr^{-n}, where G is a constant and r is the distance between them. Now, if the index n of the law

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of force exceeds 5, Chapman and Enskog showed that the effect occur
otherwise it does not.

These effects can be summarized in this way. Diffusion of matter occurs
response primarily to a concentration gradient. But it can also result fro
a temperature gradient—a phenomenon usually called the Soret effect
liquids and thermal diffusion in gases. Thus a temperature gradient can cau
a concentration gradient. Conversely, flow of heat primarily occurs in respon
to a temperature gradient. But it can also be caused by a concentration gradie
—a result known as the Dufour effect. When this happens a temperatu
difference is set up.

So far the Dufour effect (with which alone we shall be concerned) has bee
shown experimentally only for gases, where it would be expected on theo
etical grounds to be very much greater than for liquids. As a matter of fa
it reaches quite astonishing magnitudes in the case of gases. Clusius ar
Waldmann (1942) experimenting with a relatively crude apparatus got th
results shown in Table I. These are of quite modest magnitudes, but ca
culation from more precise experimental data indicates that the effect ca

TABLE I

Interdiffusing gases	Observed temperature changes
H_2—CO_2	+0·15° C. in H_2 −0·63° C. in CO_2
H_2—N_2	+0·17° C. in H_2 −0·65° C. in N_2
N_2—CO_2	+0·029° C. in N_2 −0·063° C. in CO_2
N_2—O_2	+0·017° C. in N_2 −0·019° C. in O_2

be much greater. For instance, when hydrogen at atmospheric pressu
diffuses into nitrogen at the same pressure a temperature difference of no le
than 8° C. can be produced. The difference between the result actual
observed by Clusius and Waldmann and this value they attributed to th
arrangement of their resistance thermometers and diaphragm, and to th
difficulty of siting them at the seat of the maximum temperature disturbance
This seems more than likely, especially as during the course of the prese
work results have been obtained which rough calculation has shown to be
the order of 4° or 5° C., without any special endeavour being made to loca
the maximum values.

Use of the Dufour effect. The application of the effect to the measurement
the stomatal opening of leaves is in principle very simple. A leaf such
wheat is chosen which has about equal numbers of stomata on both surface
A small jet of hydrogen is then blown on to one side of the leaf. Immediatel
opposite the jet on the other side is placed a fine thermocouple connected
a sensitive galvanometer. The hydrogen diffuses through the stomata an
across the leaf at a rate which varies with the degree of stomatal opening, an

onsequently the indication of the galvanometer changes as the stomata open r close.

In practice, however, the matter is not quite so simple. Obviously, if the nstrument is sound in principle it ought to give a zero indication when an npervious membrane—such as a piece of mica or a microscope cover-glass –is placed in the position of the leaf, and for a long time this very desirable esult could not be achieved. Why this was so can be seen by considering what appens when the hydrogen jet is directed on to the leaf (Fig. 1).

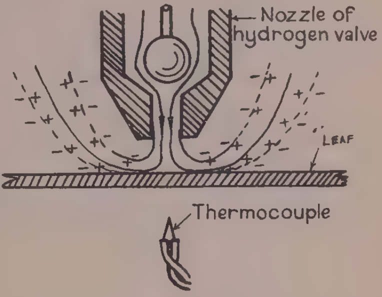

FIG. 1.

At the boundaries of the jet interiffusion of hydrogen and air will be ccurring. On the hydrogen side of he boundary the temperature will be aised; on the air side it will be owered. Now even if the leaf is quite mpermeable these temperature changes will be transferred by conduction and will influence the thermocouple, and his was in fact invariably found to be he case. Several ways were tried of minimizing this trouble—such as directing he jet into a little glass open-bottomed cup fixed to the leaf—but none was atisfactory. It was also suspected that the temperature of the jet-pipe might e influenced by the proximity of cooled air, but attention to this detail was no more fruitful of success.

The difficulty was eventually overcome by making use of the fact that the various transport processes involved occur at different speeds. It can be shown that a temperature disturbance is conducted through a medium at a rate which depends, so far as the medium influences it, on the magnitude of $\sqrt{(K/\sigma\rho)}$, where K is the heat conductivity of the medium, ρ is its density, and σ is its specific heat. On the other hand, the rate at which diffusion of, say, hydrogen takes place under analogous conditions is measured by $\sqrt{D}$, where D is the coefficient of diffusion of hydrogen in the medium. Now if we compare the relative rates of propagation of a temperature wave through the water of the leaf, and through air, with that at which a hydrogen wave diffuses through air we get the following results:

Temperature wave in water $K = 0{\cdot}00143$ cal./sec./° C./cm.
$\sigma = 1{\cdot}0$ cal./g./° C.
$\rho = 1{\cdot}0$ g./cm.^{3}
$\sqrt{(K/\sigma\rho)} = 0{\cdot}038$
Temperature wave in air $K = 0{\cdot}000057$ cal./sec./° C./cm.
$\sigma = 0{\cdot}24$ cal./g./° C.
$\rho = 0{\cdot}00121$ g./cm.^{3}
$\sqrt{(K/\sigma\rho)} = 0{\cdot}44$
Hydrogen wave in air $D = 0{\cdot}634$ cm.^{2}/sec.
$\sqrt{D} = 0{\cdot}80$

that is, the rates are in the ratios $1 : 11\frac{1}{2} : 21$.

It seemed as a consequence of these calculations that it would be worth while to work with transient effects rather than steady ones and to try to pick up the hydrogen wave before the conducted temperature wave had got across —to put the matter rather simply. Clusius and Waldmann indicate, on the basis of Enskog's theory, that the maximum temperature difference in the interdiffusion of gases occurs after an interval given roughly by

$$t \approx l^2\frac{\sigma\rho}{K},$$

where l is the distance from the original plane of separation to the point in question and K, σ, and ρ have the same meanings as before. This means that with a thermocouple, say, 2 mm. from the leaf the maximum e.m.f. might be expected to occur after an interval of about $\frac{1}{5}$ sec. Again, if we assume for simplicity that along a given plane in air the hydrogen concentration is made to vary harmonically with a frequency f, then diffusion waves of hydrogen will move through the air with an actual velocity of

$$V = \sqrt{(4\pi f D)}.$$

For the sake of argument let $f = 10$ per second. Then the velocity works out at about 8·9 cm. sec. and such a wave will have reached the thermocouple distant 2 mm. from the plane, in a time of the order of $\frac{1}{50}$ sec.

These approximate calculations seem to indicate that to distinguish between the temperature disturbances at 2 mm. from the leaf caused on the one hand by the diffusion of hydrogen through the leaf and on the other hand by the conduction through the leaf of temperature changes originating on the far side a thermocouple and galvanometer of very rapid response would be required. This in fact proved to be the case, but before describing the apparatus which was finally constructed it may be of interest to mention the relation between the magnitudes of rapid changes in the air temperature and the corresponding changes caused in the thermocouple. It will, of course, be at once recognized that a thermocouple cannot follow exactly rapid temperature fluctuations in the air around it. The heavier the couple the less of the temperature variation does it pick up. The exact extent of its response, however, may be evaluated as follows. Suppose that the temperature of the air around the thermo-junction varies harmonically with an amplitude α and a frequency f, i.e. the air temperature is given by an expression of the form

$$T = T_0+\alpha \sin 2\pi f t,$$

where t stands for time. Then it can be shown that for small circular thermocouple wires of radius a, specific heat σ, and density ρ, the amplitude of their temperature variation is given by

$$\beta = \frac{\alpha}{\sqrt{\left\{1+\left(\frac{\pi f a\sigma\rho}{\epsilon}\right)^2\right\}}},$$

where ϵ is the emissivity of the surface of the wire, i.e. the rate of heat transfer or from the wire per unit area of surface per degree difference in tempera-ure between it and the air. Taking the diameter ($2a$) of the wires as $5\,\mu$, f as per sec., $\sigma = 0{\cdot}0294$ cal./g./° C., $\rho = 9{\cdot}80$ g./cm.^{3}, and $\epsilon = 3{\cdot}0 \times 10^{-4}$ l./sec./° C./cm.^{2} we get the result

$$\beta/\alpha = 1/7{\cdot}6,$$

e. the wires succeed in picking up one-eighth of the temperature changes ctually occurring. This serves to emphasize how necessary it evidently is to se a really fine thermocouple, for if the elements had been of ordinary fine ire, gauge 50 for instance, the fraction of the temperature variation picked p would then have been only about one-fortieth.

Design of the instrument. In designing an instrument to work on these rinciples there are several obvious requirements: (i) The atmosphere on one ide of the leaf must be changed locally as rapidly as possible from pure air pure hydrogen. The easiest way of doing this would seem to be to direct he hydrogen on to the leaf as a jet and to have the valve controlling it as close the leaf as possible. (ii) The thermocouple must be of extremely fine wires, nd rigidly positioned with respect to the leaf. (iii) The galvanometer must ave a period of preferably not longer than 0·1 sec., and the sensitivity must e very high. It must be critically damped to gain the fullest advantage of its peed. (iv) The jet should be directed *downwards* on to the leaf. Experience howed that otherwise it flowed round the leaf as it rose and a deflexion was btained even when a mica strip replaced the leaf, unless indeed the strip vas made much wider than the leaf it replaced. (v) The hydrogen should be upplied at a constant head, and this should be low. This latter circumstance nsures that it does not build up to any appreciable pressure when it impinges n the leaf—its passage through the stomata is then purely by diffusion.

Construction. The general construction of the instrument finally developed s shown in Fig. 2. The hydrogen valve was made of perspex. It was operated y a small electromagnet which pulled the steel ball closing the lower end ff its seating. The design of this valve ensured that the hydrogen first eaching the leaf was not contaminated with more than a trace of air. Supported n the stem of the valve was a clip of gauge 20 spring wire. The jaws of this pened when it was compressed, and they were formed of two shallow glass rings with ends ground flat. These served to grip the leaf, and incidentally the upper one also deflected the hydrogen upwards clear of the thermocouple after it had impinged on the epidermis. The glass rings were cemented to the wire clip with Araldite,[1] and two 44-gauge enamelled copper leads twisted together were also fixed on in the same way. These served to carry the thermo-electric wires, and they were taken to a small terminal block from whence stouter wires were connected to the galvanometer.

Details of the thermocouple are shown in Fig. 3. The elements were composed of bismuth and an alloy of bismuth with 5 per cent. tin—a combination giving the very high thermo-electric power of 108 microvolts per ° C. The

[1] A thermosetting resin obtainable from Messrs. Aero Research Ltd., Duxford, Cambridge.

wires were approximately 5 μ in diameter, and were drawn out in thick-walle capillary glass tubing in the way described by Strong (1938). They wer soldered to the bonded copper leads as follows. The ends of the latter wer first cut level with a razor-blade, and then 'tinned' with a heated copper wir of gauge 22 moistened with Wood's metal. Zinc chloride in distilled wate

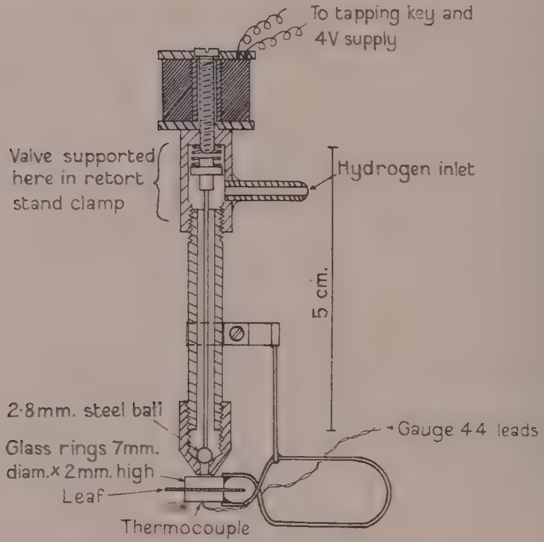

FIG. 2.

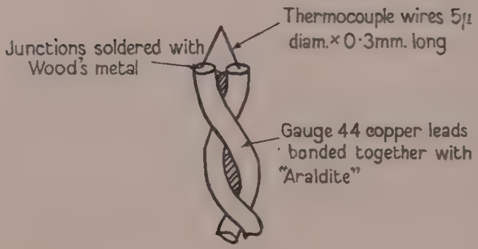
Thermocouple wires 5μ diam.× 0·3mm. long
Junctions soldered with Wood's metal
Gauge 44 copper leads bonded together with "Araldite"

FIG. 3.

was used as flux. The leads were then supported in contact (near their end with a short piece of gauge 48 Eureka wire. This could be heated by passin a current through it to just such an extent as to melt the minute pools Wood's metal covering the ends of the gauge 44 leads. In these circumstance short pieces of the thermo-electric wires held on a glass splinter in a micr manipulator with a trace of vaseline were touched end-on to the leads. A soon as they had penetrated any surface film present on the Wood's metal th latter wetted them and began to draw them in by capillary attraction. As it di of course it dissolved the wires. This raised its melting-point, and soon the ocess came to an end with the minute wires firmly fixed to their leads. The res were usually soldered in turn, not simultaneously. They were then cut level at about $\frac{1}{3}$ mm. from the junctions with the leads. A splinter of razor-ade held in the micromanipulator was found convenient for this. One eration remained, that of joining together the free tips of the wires. This s accomplished by condenser discharge. The details of the circuit did not pear critical, but a capacity of 4 mf. charged to about 10 volts was usually ed. A resistance which was first made very high (say, 2,000 ohms) was aced in series with the couple, and this was progressively reduced till a tisfactory result appeared to have been achieved. The condenser was arged up, and then observing the wires through a binocular microscope eir ends were carefully touched together with the micromanipulator. eedless to say, once a satisfactory joint had been formed the policy was to ave well alone. The resistance of the complete couple was about 80 ohms.

The 44-gauge leads of course had previously been fixed to the wire clip ar the lower of the glass rings with Araldite, and it only remained to adjust em with a needle till the thermocouple tip was a suitable distance from the se of the ring. About 1-2 mm. was the distance usually chosen. The nearer e couple is to the leaf the quicker is the sequence of temperature changes at meet it. To this extent therefore its response will be smaller. On the other nd, the farther the couple is from the leaf the more subject it is to the effects stray air currents. Moreover, it is fairly obvious that the actual maximum mperature change reached at any point will vary with the position of that oint, falling off both very near the leaf and at a distance from it. The mathe-atical investigation of the optimum conditions is very difficult, but for-nately it is a relatively easy matter to fix a good position for the couple by ial and error.

Auxiliary apparatus. The only other pieces of apparatus required were a drogen supply and a galvanometer.

The hydrogen supply consisted of a 'bell' (Fig. 4) made out of a Win-ester quart bottle with the bottom removed. This was floated in the usual ay in a longer glass vessel filled with strong brine. Its neck was attached to wire passing over a pulley to a counterweight, the object being to stabilize in a vertical position as it rose or fell, and to adjust the gas pressure within to about 2 or 3 in. of water. The rest of the details are self-explanatory.

The galvanometer was a special short-period one kindly lent by Mr. A. C. owning of University College, London, and designed and built by him. s characteristics were roughly as follows:

riod	0·1 sec.
nsitivity at 1 metre . .	30 mm. per μv.
esistance	30 ohms
ritical damping resistance .	Probably about 2,000–3,000 ohms. A resistance of 1,000 ohms. was in fact always used.

n instrument with about one-fifth of the number of turns in the coil would

have been much better for the present purpose, as it would have gained f
more from the reduction in the critical damping resistance than it would hav
lost in sensitivity. In fact its performance would probably have been improve
nearly fivefold.

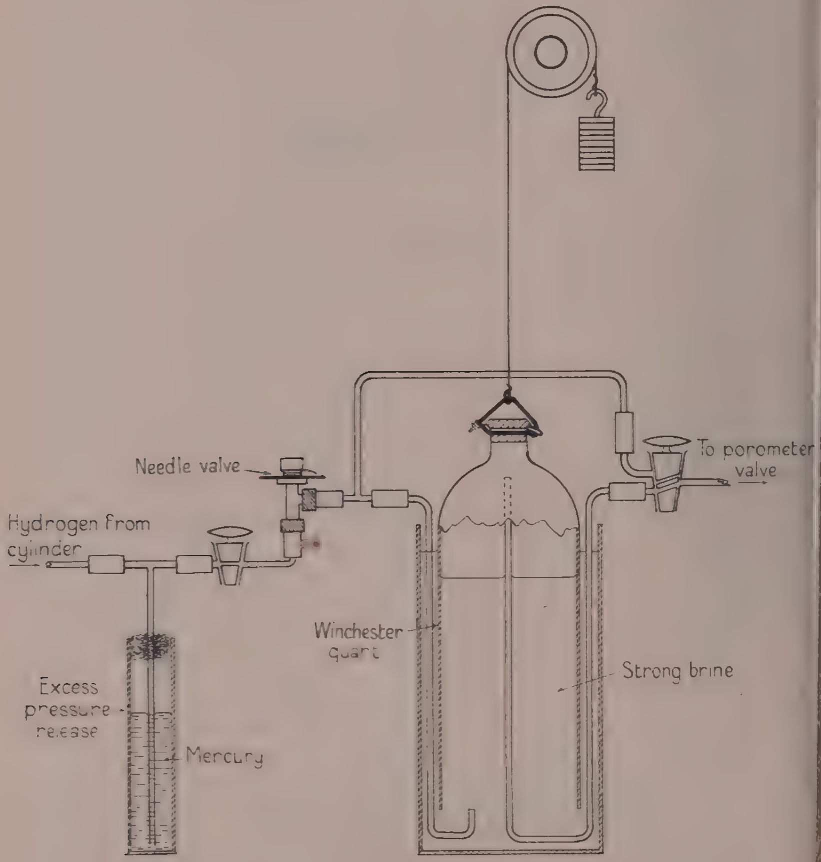

FIG. 4.

Behaviour and use of the apparatus. The behaviour of the apparatus is
follows. Suppose that a leaf or small rectangle of paper is in position betwee
the glass jaws of the clip. Then the system having been flushed with hydroge
and the galvanometer spot having returned to its steady position of res
depression of the key results in the following sequence of changes. T
galvanometer spot first flicks violently to the right (say) and then back agai
As it passes the zero it begins to slacken, and finally comes to an unstea
position of rest to the left (Fig. 5). The deflexion which is recorded is the initi
rapid 'kick', and since this occurs in less than about $\frac{1}{5}$ sec. it is unnecessa

keep the hydrogen flowing for longer than this. On closing the valve the lvanometer spot returns to rest in a relatively short time—say, 15–30 sec. repeat reading can then be taken.

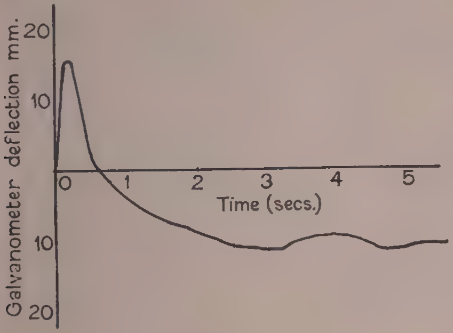

Fig. 5.

The sequence of events can be interpreted possibly as follows. Imagine two ases of nearly equal molecular weight initially separated by an impermeable embrane (Fig. 6). We can represent the temperature by the horizontal ne *AB*. Now imagine the membrane to be suddenly withdrawn. Interiffusion of gases will occur and the temperature distribution after a small terval of time will be somewhat as shown (*CD*, Fig. 7). The mean plane of

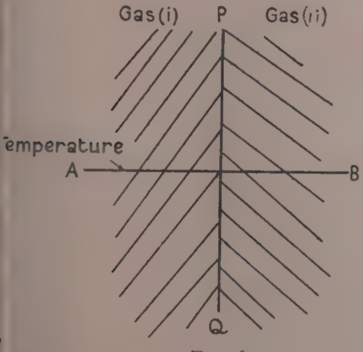

Fig. 6.

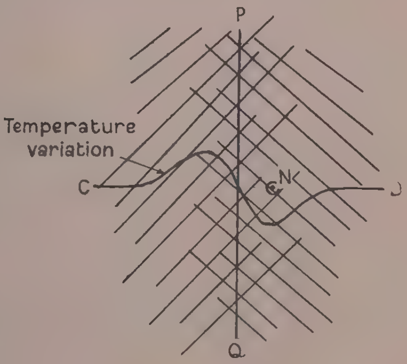

Fig. 7.

eparation of the two gases will remain at *PQ*, since the gases are of nearly qual density. A thermocouple placed at *N* would react as indicated in Fig. 8. Now the situation in the porometer differs from the one just considered that two gases of widely differing densities are involved. The molecules of he one move much faster than those of the other. To allow for this in an pproximate fashion we must imagine the mean plane of separation *PQ* to move to the right, say (gas (i) being hydrogen). Thus the thermocouple at *N* will experience first the effect of the temperature trough and then that of temperature crest. Ultimately in the model it would return to ambient te perature, but in the porometer a further factor is present in that the hydro concentration is maintained at 100 per cent. at the plane of the leaf. C sequently so long as hydrogen flows the galvanometer shows a rather unste but permanent deflexion in the opposite direction to its initial flick (Fig.

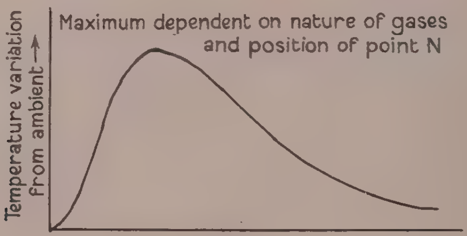

FIG. 8.

Movements of wheat stomata. In order to test the instrument in a fam setting a young wheat leaf was arranged between the glass rings. The w leaf was detached and had its lower end in water. Light from a 150-W. was concentrated on the epidermis below the jet by means of a large lan condenser lens, and in addition arrangements were made to blow two jets of moist CO_2-free air on to the upper and lower surfaces between obse tions. The leaf was cut in the greenhouse at about 12 noon and brou straight down to the laboratory. It gave no immediate indications in apparatus, so the light was switched on and the two CO_2-free air jets arra to play on those areas of surface within the glass rings. Subsequently the and air were interrupted, and finally the leaf was allowed to wilt. Fig. 9 sh how the galvanometer reading varied during this time. The response fol very closely the pattern already well known from observations with the re ance porometer, but it is interesting to have this modest confirmation closing of the stomata does exert a profound influence on passage of gase *diffusion* no less than by mass flow.

It may be interesting here to record some comparable deflexions obta with various sorts of paper or card in place of the leaf:

Membrane	Galvanometer deflexion (mm.)
Mica	zero
Glazed writing-paper	33
Card-index card	14
Thin typewriter paper	31
Two thicknesses ditto	24
Three ditto	18·5
Four ditto	14·5
Five ditto	12
Six ditto	10

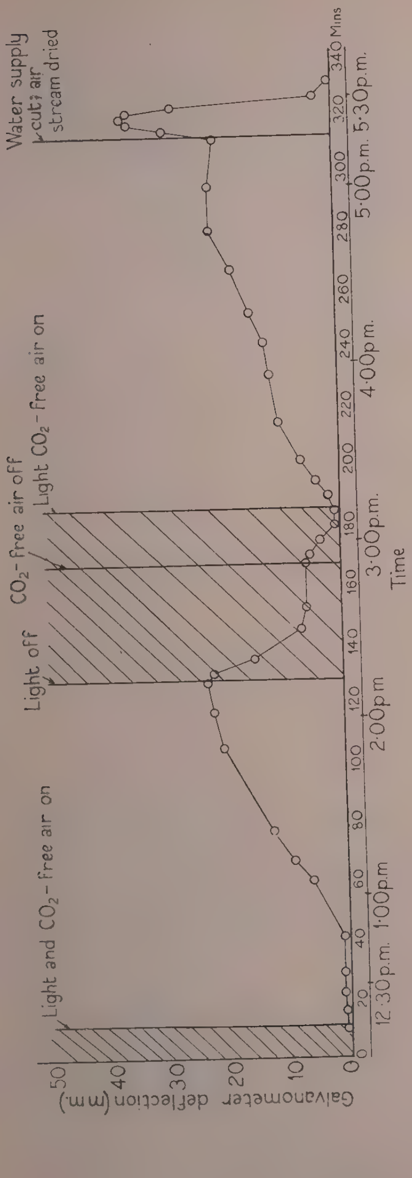
Galvanometer deflection (m.m.)
0
10
20
30
40
50
Light and CO₂ - free air on
Light off
CO₂ - free air off
Light CO₂ - free air on
Water supply cut; air stream dried
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340 Mins
12·30 p.m.
1·00 p.m.
2·00 p.m.
3·00 p.m.
4·00 p.m.
5·00 p.m.
5·30 p.m.
Time

Fig. 9.

The last set of readings is interesting. They enable a rough idea to be gained as to how closely the galvanometer readings follow the 'diffusiv conductance' which is the real goal of measurement. They can be analysed i a rough way as follows. Let X be the 'resistance' of the open air path betwee the 'leaf' and the thermocouple. Let Y be the resistance of a single thicknes of paper. Then if n is the number of sheets of paper and S is the galvanomete deflexion, the total resistance between the side of the leaf nearest the jet an the plane of the thermocouple will be $X+nY$ and this can be compared wit $1/S$. This has been done in Fig. 10, from which it can be seen that th

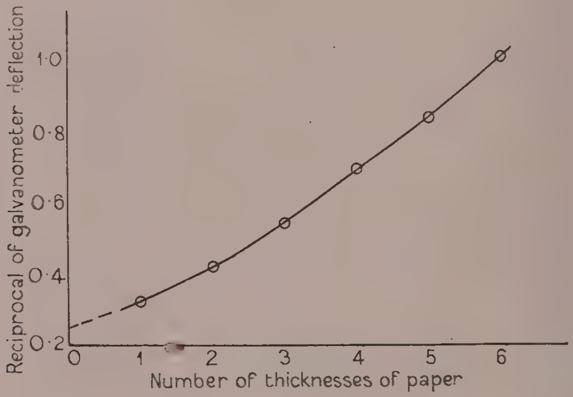

FIG. 10.

relation is not strictly a linear one. This means that S is not strictly a measu of the total conductance of the path, at least when the 'leaf' thickness variable. Probably alteration of the thickness changes the 'pattern' of t temperature waves, but the subject needs looking into further.

Conclusion. In conclusion, it might be helpful to summarize the advantag and disadvantages of the new method. The advantages are: (i) A diffusi process is employed, and the answers are therefore more likely to be in a for at once useful in questions of transpiration and assimilation. A certain exter diffusion path is, however, included with the leaf. (ii) No part of the leaf required to be even temporarily enclosed, and the use of washers is avoide (iii) The instrument can very easily be made automatic and recording. (i Ambient temperature changes are not troublesome, unless comparable rapidity with the galvanometer 'flick'.

Disadvantages are: (i) Only leaves having stomata on both surfaces suitable. (ii) The construction of the thermocouple requires a certain amou of skill, though this is easily acquired. (iii) A special type of galvanometer usually available is needed.

It might perhaps be added at this point that the existence and magnitu o the obscure effect which has been the subject of this paper may prove to be a listurbing factor in previously described instruments, in particular in the dfusion porometer (Gregory and Armstrong, 1936).

It is a pleasure to record my thanks to Mr. F. S. Smith for the care with ich he made several very necessary parts of the apparatus; to Dr. O. V. S. eath for his interest and encouragement; and to Mr. A. C. Downing of iversity College for the loan of a very fine galvanometer.

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Phosphorus utilization by *Asterionella formosa* Hass.

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SUMMARY

Observations have been made on the relationship in Lake Windermere between the growth of *Asterionella formosa* and the concentration of dissolved phosphate. *Asterionella formosa* has also been grown in culture and the amount of phosphorus required by this organism has been determined. These experiments have shown that:

(1) *Asterionella* can take up and store in reserve phosphorus from concentrations below those found in phosphorus-poor lakes (i.e. below 1 μg.P/l.).
(2) Growth can continue in phosphorus-deficient media by making use of such reserves, cell phosphorus being steadily reduced.
(3) The limiting requirement per cell of phosphorus is very minute—about 0·06 μg P/10^6 cells, so that initial concentrations as low as 1·0 μg.P/l. can theoretically produce a population of some 16 × 10^6 cells/l. before limitation by phosphorus deficiency. This has been realized in culture.

The behaviour of *Asterionella formosa* growing in nature has been found to conform with that found in culture.

It is concluded from such observations that phosphorus deficiency is unlikely to provide a limit to growth of *Asterionella* in Windermere, despite the very low initial concentration of dissolved phosphate.

Further experiments have shown that *Asterionella* cells low in phosphorus can rapidly take up added phosphate from lake water but not from distilled water. Some factors which affect the rate of phosphate uptake of depleted cells are investigated, and attempts have been made to throw some light on the nature of the apparent difference between lake water on the one hand and distilled water and a number of artificial lake waters on the other. No conclusion is reached on the reason why *Asterionella* cells behave differently in lake water and in artificial media.

INTRODUCTION

FOLLOWING the work of Deniges (G. Deniges, 1921), many limnologis have measured the concentration of phosphate phosphorus in solution in great number of natural waters. These investigations have usually confirme the widespread scarcity of phosphorus in solution both in the sea and in fres waters, with a concentration range in the latter from 'zero' to some 20 μg.P and occasionally somewhat higher (Atkins, 1923; Juday, Birge, Kemmere and Robinson, 1928; Juday and Birge, 1931). This widespread scarcity phosphorus, together with observations showing a marked correlatic between the rise in algal populations and fall in phosphorus concentratio have sometimes suggested that the growth of algal populations in natu might be limited by shortage of phosphorus (Atkins and Harris, 192.

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Yoshimura, 1932). However, a great deal of data exists which, while conirming that phosphorus normally occurs in solution in extreme dilution, hows no correlation whatever between phosphorus concentration and the ise and fall of algal populations (Juday, Birge, Kemmerer, and Robinson, 1928; Juday and Birge, 1931).

Measurement of the dissolved phosphate in Lake Windermere has been made since 1946 together with counts of the dominant algal species (*Asterionella formosa*). These analyses have shown that the concentration of phosphate phosphorus in Windermere varies from below 1·0 μg.P/l. to a maximum of 2 μg.P/l. and bears no relationship to the population of *Asterionella*. This result is similar to those described by Juday, Birge, Kemmerer, and Robinson. It is strange that high initial concentrations of dissolved phosphorus are often associated with a marked fall in phosphate concentration as algal populations ncrease (Gardiner, 1941), while low initial concentrations of phosphate show no such correlation.

As no general conclusion can be drawn from the literature concerning the role of dissolved phosphorus as a limiting factor in the growth of algal popuations, the present study of phosphate utilization by *Asterionella formosa* was made.

EXPERIMENTAL METHODS

Sampling. Water samples for algal counts and for chemical analysis consisted of ntegrated samples taken from the surface 5 metres of the lake. These were obtained by lowering a 5-metre length of rubber hose of approximately ½ in. internal bore and weighted with lead at the lower end into the water from a boat. As the hose was owered with the weighted end foremost into the water, a column of water 5 metres ong entered the tube. When the tube was fully extended, the upper end was closed with the thumb, and the lower end raised by means of a cord attached to the weight, so that the water sample was retained in the tube and then poured into a suitable container. This method of sampling was devised by Lund (1949).

Samples of algae for analysis were obtained by towing a phytoplankton net of bolting silk (180-mesh) behind a boat until sufficient material for analysis had been caught. The length of tow required depended of course on the density of the popuation, but for each determination of cell phosphorus about 10^6 cells were required. If many particles of debris and other organisms apart from *Asterionella* were present n the water, it was necessary to separate the *Asterionella* as much as possible.

It was thought to be essential to separate zooplankton organisms at the time of collection rather than later in the laboratory, as it has been shown here and elsewhere (Gardiner, 1937) that zooplankton organisms liberate large quantities of phosphate into the surrounding water, and it seemed likely that if a mixed collection of zoo- and phytoplankton was made, the phosphorus content of the phytoplankton could easily be altered by the excreted phosphate from the zooplankton.

When large populations of zooplankton were present a coarse mesh net (mesh 60) was fastened over the mouth of the phytoplankton net so that zooplankton were never in contact in large concentration with the phytoplankton. If necessary, eparation of the *Asterionella* from other algae, particularly *Melosira*, and debris could then be effected in the laboratory by filtering the algal suspension with suction through a course sintered glass filter. This procedure allowed only a proportion of the *Asterionella* cells to pass, but stopped most of the *Melosira* and a grea deal of the larger particulate matter. As the growth maximum for *Asterionella* was approached in the lake, such separations were usually unnecessary as the populatio was predominantly *Asterionella*.

Cell counts were made, using the inverted microscope as suggested by Utermöh (1931). The counting chambers were made from glass tubing (2 cm. int. diam. an 5 cm. high). To one end of the tube a cover slip was cemented, using polystyren cement. In this way cylindrical vessels were constructed of about 15 ml. capacity For counting, a quantity of sample such that it contained about 100 colonies o *Asterionella* was pipetted into one of these tubes, and the cells precipitated b addition of a few drops of a strong solution of iodine in potassium iodide. In orde that the total number of colonies in the tube should be about 100, it was sometime necessary to dilute the sample of cells with distilled water. After 3 hours the cell had sedimented on to the cover-slip base of the tube, and the entire area of th cover-slip was examined with the inverted miscroscope and the total number of cell counted. The number of cells per litre of the original sample could then easily b calculated. A count of about 100 colonies has been taken to give reasonable accurac (Lund, 1949).

Dissolved phosphate. The method used was that of Atkins practically withou modification (Atkins, 1925). 100-ml. samples of the water were taken and to thes 1 ml. of acidified ammonium molybdate solution (100 ml. 10 per cent. ammoniu molybdate mixed with 300 ml. 50 per cent. H_2SO_4) was added, followed by 4 drop of stannous chloride solution (2·5 g. $SnCl_2$ dissolved in 10 ml. conc. HCl made u to 100 ml. with distilled water and filtered). The stannous chloride solution wa kept in an all-glass hypodermic syringe of 20-ml. capacity, the tip of which had bee drawn out to form a narrow capillary. A small piece of tinfoil was kept inside th syringe, which contained no air-bubbles. In this way drops of stannous chlorid were obtained without exposing the reagent to air, and as no oxidation could tak place the solution could be kept indefinitely. The blue colour which developed i the sample in the presence of phosphate was compared with standard solutio similarly treated, in long glass nessler tubes (32 × 2·3 cm.) with sealed-on flat base using a 'daylight' fluorescent lamp below the tubes for illumination. Sever standards were made by adding known amounts of standard ammonium phosphat solution to distilled water, so that a standard very close in concentration to t sample could be chosen; colours were compared 5 minutes after addition of reagent In this way as little as 1 μg.P/l. could be detected and approximately determine but a greater concentration was necessary for accurate analysis.

Cell phosphorus in culture. Phosphorus contained by the cells was estimated b filtering 1 litre samples of the culture medium through a small Whatman No. 5 filter, washing the cells briefly on the filter with distilled water, and then puncturi the filter-paper and washing the cells into a small flask so that most of the *Asteri* *nella* from 1 litre were concentrated into about 30 ml. of distilled water. From th concentrate a proportion, depending on concentration of the cells, was removed f dilution and counting, to find the number of cells per ml. contained in the distill water suspension. The remaining suspension was then pipetted into duplica 150-ml. silica flasks and evaporated to dryness. The residues were oxidized by means perchloric acid. 1 ml. of *c.* 20 per cent. solution of perchloric acid (P free) was add to the residue and the flask warmed over a bunsen burner; water was first driven o nd the cells then began to char. Heating was continued carefully until the liquid ecame quite clear, and the residue allowed to cool. It was sometimes necessary to ool and reheat the perchloric acid mixture several times before it was perfectly olourless. The oxidized residues were then diluted to 100 ml. and the normal olorimetric procedure for phosphate determination applied. It was found to be nnecessary to remove excess perchloric acid as this acid, although powerfully xidizing when concentrated, has little oxidizing ability when diluted. The above rocedure gave satisfactory and reliable results, but when the number of cells for ach analysis was very large (e.g. when the culture contained a great many cells of w P content) it was sometimes necessary to filter the extract obtained after xidation and partial dilution through a washed Whatman 541 to remove the silica ustules of the diatoms which otherwise caused cloudiness and rendered the subseuent colorimetric determination of P difficult.

Cell phosphorus in natural populations was estimated in a manner similar to that escribed for cultural cells. In this case the cells from a quantity of lake water, after eparation from debris, were concentrated by filtration, washed into suspension in istilled water, and the concentration in this suspension determined by diluting a easured portion and counting. At least 10^6 cells were required for each analysis, nd analyses were made usually in duplicate.

'*Organic phosphorus*' in solution was estimated by a similar procedure. Suspended atter, including cells, was removed from the sample by filtration through Whatman 41, and 100-ml. samples of the filtrate evaporated to dryness in 150-ml. silica asks. The residue was then oxidized with perchloric acid and treated in the manner escribed for cell phosphorus. This gave the total of dissolved and 'organic phoshorus'.

Laboratory Cultures. Cultures of *Asterionella* were made using 20-l. flasks (round lat bottom) as culture vessels. The culture media consisted of water obtained from Lake Windermere from 50 metres depth, to which only silica was added to a concenration of approximately 20 mg./l. of SiO_2. It was necessary to add silica because previous work on *Asterionella* in Windermere and other lakes has shown that in these waters lack of silica normally controls the extent to which *Asterionella* can grow, there being enough SiO_2 present in normal years (2·2 mg./l. in Windermere) o produce *c.* 8×10^6 cells allowing for wastage and a final unusable SiO_2 concenration of *c.* 0·5–0·25 mg./l. SiO_2. Each million cells of *Asterionella* require an almost constant amount of SiO_2 equivalent to 0·14 mg. $SiO_2/10^6$ cells. No other additions were made to the cultures, and they contained an initial concentration of soluble phosphate of 1·0 μg.P/l. and 6·0 μg./l. of 'organic' phosphorus. The 20-l. flasks were plugged with cotton, through which a siphon tube was passed for sampling, and provision made for stirring by blowing air through the culture. The flasks were rendered free from algal contamination (but not necessarily free from bacteria) by heating to 100° C. The flasks were inoculated with *Asterionella* which had been grown in Chu medium (Chu 1942) without phosphorus (normal Chu contains an enormous P concentration), to give an original cell concentration of *c.* 100,000/l.

The flasks were illuminated by four 250-watt lamps arranged concentrically, with a sheet-metal circular reflector placed outside the lamps to increase illuminaion. In order to prevent overheating the cultures, the flasks were cooled by a water jet applied to the top of the slightly tilted flask so that a film of water flowed over the surface and ran away at the base; this film is quite regular if the glass is clean and does not materially decrease the illumination. Growth normally took place under these conditions at the rate of one division per day, although several unaccountable failures were experienced when the cells died under conditions identica (as far as could be ascertained) with those stated above.

During growth the cell numbers were counted regularly together with analyses o total phosphorus contained by the cells, soluble phosphorus, and 'organic' phosphorus in solution. Uniformity in the culture was originally maintained by passin air through the liquid at the rate of about 3·5 l./hr., but in later experiments thi procedure was thought to be unnecessary and was discontinued in favour of occasional shaking, which was found to preserve a uniform suspension of cells.

EXPERIMENTAL RESULTS

Field observations. Measurements of the concentration of dissolved phosphorus in Lake Windermere (and other lake-district lakes) have been mad regularly since 1946.

The concentration of phosphorus in Windermere (which is typical of man of these lakes) is always low, fluctuating between a maximum of 2 μg.P/l. i winter and a minimum below 1 μg.P/l. in summer. Growths of *Asterionell formosa* of the order of 10^7 cells/l. are normally produced in the spring of eac year, and there is little correlation between this growth and the small change in phosphate concentration, in contrast to the marked correlation found b Gardiner (1941) between the growth of *Asterionella* and the very large fall i phosphate concentrate, the latter being initially as high as 66 μg./l. Typica measurements of phosphate concentration and algal numbers are plotted fo Windermere in Fig. 1, together with some data from Gardiner's paper fo comparison. It is clear that the *Asterionella* cells in Windermere must use very much smaller amount of phosphorus in their growth than those in th reservoir studied by Gardiner.

Measurements of the actual phosphorus content of *Asterionella* cells mad at the time of maximum growth in Windermere and other lakes show tha this quantity is somewhat variable, but usually about 0·4 μg.P/10^6 cells, s that a population of 10^7 cells/l. contains phosphorus equivalent to 4 μg.P/l It is not possible therefore to account for the fall in phosphate concentratio of 64 μg.P/l. observed by Gardiner without postulating that the cells in hi reservoir contained a very much larger amount of phosphorus.

On the other hand, it was difficult to explain the fact that the Windermer cells contained the equivalent of 4 μg.P/l. while the water had an initial concentration of phosphate of only 1 μg.P/l.

It seemed possible that additional phosphorus could be obtained by th cells by utilizing the 'organic' phosphorus contained in many natural waters This phosphorus occurs in solution in a form which is not detected by th method used for measurement of dissolved phosphorus, but may be determined after oxidation of the residue left on evaporation of the water. Th chemical form in which this phosphorus is held is not known (Juday an Birge, 1931; Hutchinson, 1941).

Alternatively, phosphorus was known to occur in bottom deposits i latively large amount (Mortimer, 1941–2; Einsele, 1936, 1938), possibly dsorbed on to ferric hydroxide which forms a part of many muds. Such ccumulations are released on raising the pH of the water above the mud or n reducing the ferric compounds to the ferrous state. On the other hand, ssolved phosphate must be in equilibrium with adsorbed ions, so that if osphate is removed from solution by any agency, more might be expected pass into solution to maintain equilibrium.

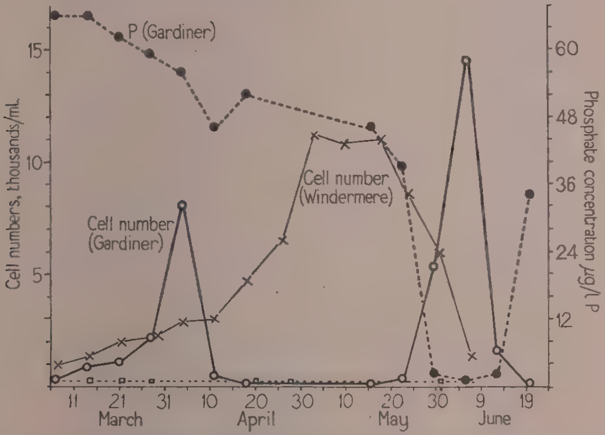


FIG. 1. Data taken from Gardiner (1939) are plotted to show the marked fall in phosphate concentration (– – ● – –) which accompanies the rise in *Asterionella formosa* population (—o—) in Island Barn reservoir. In contrast some findings from Windermere show a rise in *Asterionella formosa* population (—×—) unaccompanied by any great change in phosphate concentration (– – □ – –), as the starting concentration of phosphate is very much lower than that in Island Barn reservoir.

Measurement of the concentration of phosphate in equilibrium with both nud and synthetic deposits of ferric hydroxide showed this to be about μg.P/l. at 6·8–7·0, and rising with rising pH (see Fig. 2, where concentration f phosphate in solution in contact with mud is plotted against pH).

It was not known which, if any, of these sources of phosphorus were tilized by *Asterionella* in Windermere, and culture experiments were underaken to obtain more information about the actual phosphorus requirements f *Asterionella*.

Results of culture experiments. The information obtained from the culture xperiments described may be summarized as follows.

1. Provided that silica was added, growth of *Asterionella formosa* would ontinue in water from Windermere Lake until a population was reached pproximately twice as great as that normally produced in the lake itself (16 × 10^6 cells/l. in culture as compared with 8 × 10^6 cells/l. in the lake), in oth cases the initial concentration of phosphorus being 1·0 μg.P/l. (as orthophosphate).

2. The concentration of 'organic' phosphorus remained unchanged in th cultures even when the phosphorus content of the cells was very much reduced. It seems unlikely that *Asterionella* can use the 'organic' phosphoru directly, although this phosphorus may, of course, be utilized over periods o time greater than those here considered (14 days).

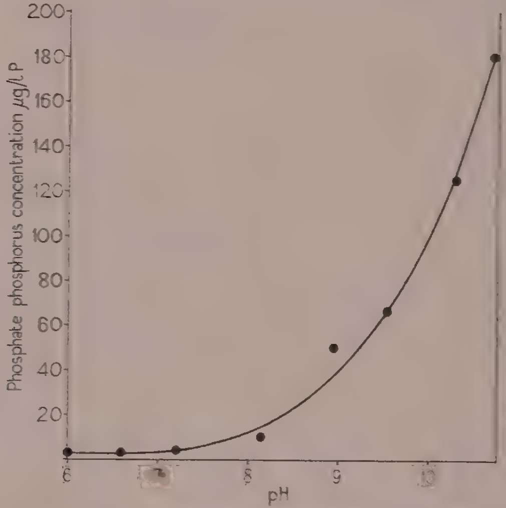


FIG. 2. The concentration of phosphate phosphorus in equilibrium with lake mud at various pH. Approximately 250 ml. of soft surface mud were suspended in 3·5 l. of water.

3. *Asterionella* rapidly absorbed practically all the phosphate from solutio reducing the phosphorus concentration to below the limits of measurement usually less than 1 day after the start of the culture.

By comparing the amount of phosphorus contained in the cells soon aft the start of the experiment with that originally available the final concentr tion of dissolved phosphorus was found to be below 0·1 μg.P l., or less th one-tenth the concentration found normally in the phosphorus-poor lakes the English lake district.

4. The phosphorus content of *Asterionella* cells in culture was found vary in a regular manner, being dependent on the amount of phosphor available and the number of cells between which it must be shared. At t start of culture experiments the phosphorus content of the cells was alwa high, 1·5 μg. 10^6 cells being a typical figure, and this phosphorus represent practically the whole of the initial dissolved phosphate. As growth proceede no more phosphorus being available from solution, the cell content of th element fell progressively, the phosphorus being shared by a greater a greater number of cells, until an apparently limiting figure for cell phosphor content was reached at about 0·06 μg. 10^6 cells. The initial absorption

osphorus from solution by *Asterionella* cells is not prevented by low con- ntrations of phosphate in the medium, as this accumulation occurs with ternal phosphate concentrations as low as 0·1 μg.P/l., nor is it prevented by n initial ample supply of phosphorus within the cells. In one experiment cells ving a phosphorus content of 0·5 μg.P/10⁶ cells were placed in a culture lution containing 1·0 μg.P/l., when the cell phosphorus was observed to se to an initial figure of 1·0 μg.P/10⁶ cells, before any detectable growth took ace.

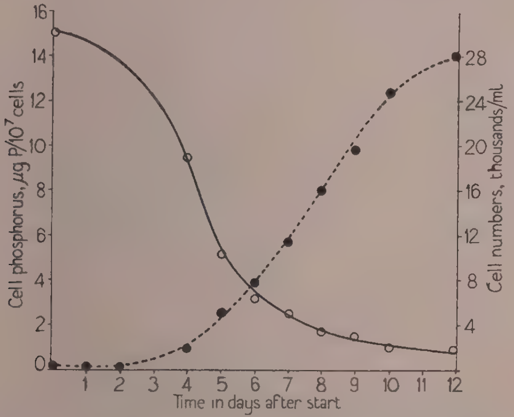


FIG. 3. Culture experiment. Cell numbers (broken line) and cell phosphorus content (unbroken line) are plotted against days duration of experiment.

5. When the cell phosphorus falls to the limiting value suggested of ·06 μg.P/10⁶ cells, growth ceases, but the addition of a further supply of hosphorus to the medium brings about a rise in cell phosphorus and re- ewed growth until the limiting cell phosphorus figure is again reached, when rowth ceases once more. These results are presented graphically in Figs. 3 nd 4.

The figure of 0·06 μg.P/10⁶ cells suggested as the lowest amount of phos- horus on which cell division can take place compares well with a similar gure quoted by Rodhe (1948), obtained indirectly, of 0·055 μg.P/10⁶ cells.

Further field observations. The behaviour of *Asterionella* cells in culture escribed above made it desirable to examine natural populations in a similar anner. Samples of the populations of *Asterionella* in Lake Windermere were ken at intervals throughout the growth period in 1950 and 1951 by means of e double net described in the section on methods. These cells were exam- ed for phosphorus content in the same manner as in the culture experiments. n Figs. 5 and 6 the changes of cell phosphorus content with rise in popula- on are plotted.

It is seen that the cell phosphorus content varies in the *Asterionella* of Vindermere in much the same way as in culture. The spring outburst of growth was commenced with cells having very high phosphorus contents– 7·0 μg.P/10^8 cells in 1950 and 0·77 μg.P/10^8 cells in 1951. As growth proceed the cell phosphorus fell progressively, reaching a minimum in 1950 · 0·23 μg.P/10^8 cells, and 0·09 μg.P/10^8 cells in 1951.

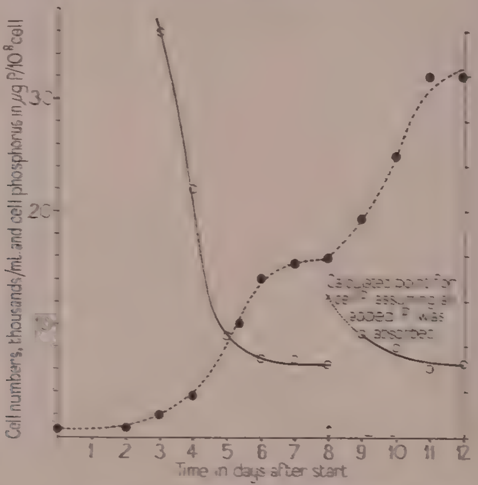
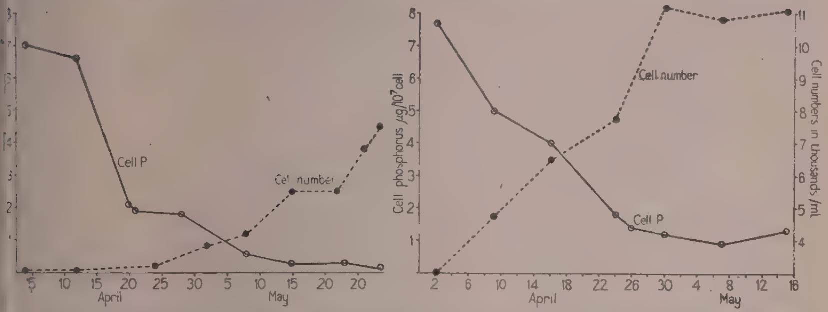


FIG. 4. Culture experiment. Cell numbers (broken line) and cell phosphorus content (unbroken line) are plotted against days duration of experiment. The point marked with an arrow at day 8 indicates the addition of a further 1 μg.P/l. as phosphate (equivalent to the initial phosphate concentration). The point on the cell phosphorus curve marked with a cross is the calculated cell phosphorus content, assuming that all added phosphorus was taken up before further cell division took place.

Had not the depletion of silica in Windermere in 1951 brought abo cessation of growth, it seems likely that phosphorus may soon have limit production in this instance. However, the conditions obtaining in Winderme in 1951 were abnormal with regard to the density of *Asterionella* populatio which reached 11,000/ml., a figure never before recorded. This large produ tion of cells took place with an initial phosphate concentration in the water approximately 1·0 μg.P/l., and a final concentration too low to measure, b a population of 11,000/ml. with a cell concentration of phosphorus 0·09 μg.P/10^8 cells would require a total amount of phosphorus in the ce equivalent to 0·99 μg.P/l., or very nearly the whole of the phosphorus initia contained in the water in solution as phosphate. This was, however, the or occasion observed by the author when *Asterionella* cells in nature approach the limiting low level of cell phosphorus found in culture.

The variability of cell phosphorus content in a phosphorus-poor mediu would also be expected to produce variability in the ratio of Si/P as the silic ntent of *Asterionella* cells is nearly constant. This expectation is realized in ractice (see Fig. 7, which presents the change in Si:P ratio with time during e spring growth period in Windermere). Clearly no calculation should be ade assuming a fixed ratio of Si:P in a phosphorus-poor medium.



IGS. 5 and 6. The variation of cell phosphorus content (—o—) of natural *Asterionella formosa* om Windermere compared with the rise in *Asterionella* population (– – ● – –). Fig. 5 gives 1950 data and Fig. 6 1951 data.

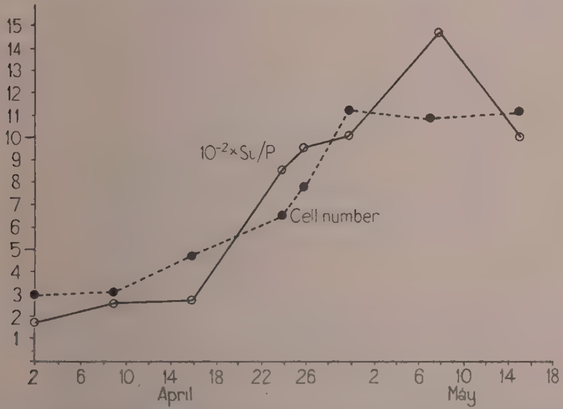


FIG. 7. Seasonal change in SiO_2:P ratio and number of *Asterionella* cells, Windermere, 1951. Ordinates give cell numbers in thousands/ml. and $[SiO_2/P] \times 10^{-2}$.

FACTORS INFLUENCING THE RATE OF PHOSPHORUS UPTAKE BY DEPLETED CELLS

It had previously been observed that cells low in phosphorus from cultures ad the ability to take up phosphorus from solution with great rapidity, as dded phosphorus disappeared from solution after a period of only a few ours. It was decided to investigate this behaviour more fully in the phoshorus-depleted cells obtained from Windermere North Basin at the end of he spring growth period.

Asterionella cells were obtained by net haul from Windermere in a practically pure state, as this species at the time constituted almost the entire phytoplankton. The cells so obtained had a phosphorus content of 0·23 μg. P/10^6 cells compared with 7·0 μg.P/10^6 cells, which was found in the early stages of the spring outburst. As a preliminary experiment, the effect of ligh on the uptake of phosphorus by these cells was investigated. A suspension o the cells obtained as above was made in distilled water and divided into equa

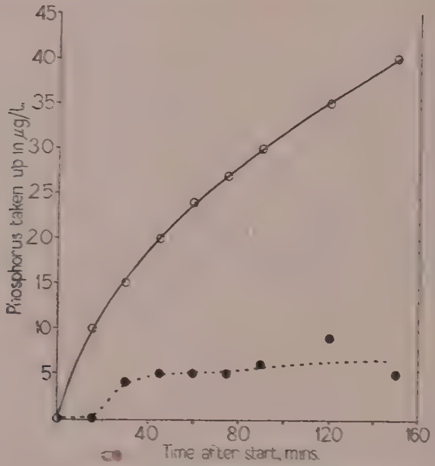


FIG. 8. Phosphorus uptake by a suspension of *Asterionella* cells taken from Windermere at the time of maximum growth in 1951. Suspensions were made in lake water (— o —) and distilled water (– – ● – –) each containing 50,000 cells/ml. and 45 μg. P/l. as phosphate.

halves. To each was added 30 μg.P/l. as ammonium phosphate, and one fla was placed in the room daylight and the other in the dark. Measurement w then made of the amount of phosphorus remaining in solution in each fla after intervals of 0·1, 0·5, 1·0, and 20·0 hours. Surprisingly, no measura amount of phosphorus was removed from solution in either light or da This result was puzzling and contrary to the tentative hypothesis which h been formed—that the rapid uptake of phosphorus by depleted cells might due to a purely physical mechanism of adsorption of phosphate on to so part of the cell tissue, either on the surface of the silica frustule or the protoplasm itself. If such an adsorption mechanism were indeed responsi for rapid phosphate uptake, it is difficult to explain why such a process co not manifest itself equally well in distilled water as lake water.

The above experiment was repeated by making a suspension of the cell lake water instead of distilled water, and taking two identical samples of suspension, adding 30 μg.P/l. as before and measuring the remaining ph phorus in solution in light and dark samples. The result was as follows.

TABLE I

The adsorption of phosphorus in lake water

Time (hrs.)	Light flask (μg.P/l. remaining)	Dark flask (μg.P/l. remaining)
0	30	30
0·1	25·5	25·5
0·3	18·0	—
0·5	18·0	18·0
2·0	2·0	2·0

It is seen that in this case the removal of phosphorus from solution was rapid and at identical rate both in light and dark.

It may now be supposed that the disappearance of the phosphorus from solution was a property of the lake water rather than the cells in suspension. The lake water was by no means free from bacteria which could have been responsible for the difference in the two sets of observations. In order to show that the seat of phosphorus uptake was indeed the *Asterionella* cells, the following experiment was made.

A cell suspension was made in lake water, containing 50,000 *Asterionella* cells ml., and in flask 1 500 ml. of this suspension was placed. A further portion of this suspension was filtered through a coarse filter to remove the suspended cells (but not the bacteria). 500 ml. of the filtrate was placed in flask 2 and the cells obtained in the filter were washed into suspension in 50 ml. of distilled water in flask 3. To each flask was added ammonium phosphate solution to a concentration of 45 μg.P/l.

The concentration of phosphate in each flask was measured at intervals. Phosphorus uptake by cells in lake water (flask 1) is shown by continuous line in Fig. 9, and that of cells in distilled water (flask 3) by the broken line. Phosphorus loss in flask 2, which is not plotted, was almost identical with that in flask 3.

It is clear from the above result that lake water containing bacteria but no *Asterionella* cells is comparatively ineffective, as also is the suspension of cells in distilled water. It is evident that both lake water and cells together are necessary for the uptake of phosphorus by phosphorus-depleted cells.

It is possible that the filtration procedure of the foregoing experiment damaged the cells in some way which rendered the cell suspension in distilled water abnormal, but this is shown not to be the case by the following experiment. A series of flasks was taken each having phosphate added to make an initial P content of 20 μg./l. They contained the water and cell suspensions listed below, and after 2·5 hours' illumination the P remaining is as indicated after each treatment listed. 1. Normal lake water containing cells in suspension (1 μg.P/l. remained). 2. Filtered lake water plus cells obtained by filtration and resuspension (1 μg./l. remained). 3. Distilled water plus suspension of cells obtained by filtration as in 2 (11 μg./l. remained). 4. Distilled water and lake mud extract plus cells in suspension (13 μg./l. remained). 5. Water from Wastwater (an unproductive lake with very low total salt content) plus cells in suspension (1 µg./l. remained). 6. Filtered lake water together with cell obtained by filtration and subsequently washed with distilled water (1 µg. l remained). 7. Distilled water plus cells washed with distilled water as used in (12 µg./l. remained).

From this result the following conclusions may be reached. (*a*) Filtration o cells from lake water does not of itself produce any damage, as the cells i flask 2 were as effective as those in flask 1. (*b*) Cells suspended in distille water are incapable of absorbing phosphorus as rapidly as those in lake wate

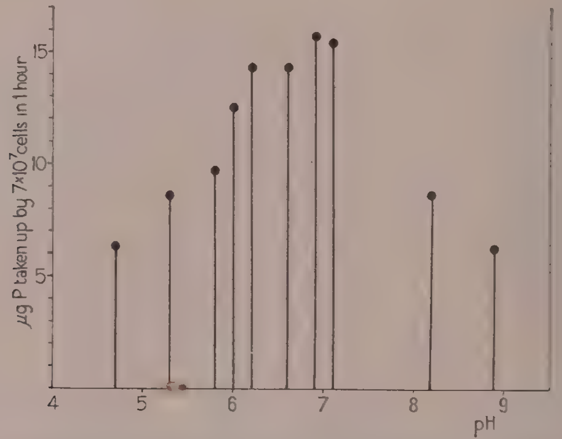


FIG. 9. Rate of phosphorus uptake per hour shown by lengths of vertical lines at different pH in lake water suspension. In each case 7×10^7 cells were suspended in 500 ml. of lake water and 50 µg.P/l. added as phosphate.

(*c*) The materials contained in a mud extract obtained by boiling mud wi water and filtration do not convey to distilled water the properties of la water from the present viewpoint. (*d*) Wastwater water in which *Asterione* does not normally grow (for reasons unknown) is as effective as Winderme water in the promotion of phosphate absorption. (*e*) Filtration of cells follow by washing with distilled water does not destroy the capacity of the cells absorb phosphate, so that apparently the inaction of distilled water is through the medium of immediate damage to the cell.

It is conceivable that the distilled water (obtained from a tin-lined cop still) contained enough toxic substance to inhibit the absorption of phospho by the cells over the period of the experiment. However, this seems unlik in view of the next experiment, in which the effect of addition of a proport of Windermere North Basin lake water to distilled water was studied. Res are given in Table II. From this it is seen that the rate of uptake of phospho from Wastwater is slightly less than that from Windermere North Basin wa by itself. It is also seen that while the uptake from distilled water is sm the uptake from distilled water plus 20 per cent. of North Basin wate

TABLE II

Phosphorus contents in μg./l. of cultures in different waters

30 μg.P/l. was added to each flask initially

Time (hrs.)	Windermere N. Basin water	Wastwater water	Distilled water	Distilled water +20% lake water	Distilled water +50% lake water
0	30	30	30	30	30
0·25	18	20	27	25·5	23
0·6	11	16	25	19	13·3
1·0	5	8	24	13	6
1·5	1·0	3·0	23	4·5	1·0

actically as rapid as from Wastwater water, while 50 per cent. of North sin water added to distilled water is practically identical with pure orth Basin water. It seems unlikely therefore that the distilled water inhibits hosphorus uptake because of any toxic quality, but it seems that the dis- tled water is in some way deficient.

A possible explanation of the different behaviour shown by cells suspended i natural waters from those suspended in distilled water was thought to lie the different ionic concentrations of the two media which may bring about motic changes which could influence the rate of absorption of ions. This ossibility was investigated by suspending cells in various salt solutions in stilled water such that the total ion concentration was similar to that in indermere water; the following solutions were made up to an initial ntent of 25 μg.P/l. and the amount of P left after 1 hour's illumination is ted in brackets: 1. Normal Windermere water (8 μg.P/l. left); 2. M/1000 aF (16·2 μg.P/l. left); 3. M/1000 KCl (21·1 μg.P/l. left); 4. M/1000 NaCl 8·2 μg.P/l. left); 5. M/1000 $CaHCO_3$ (24·0 μg.P/l. left); 6. Dist H_2O (22·2 g.P/l. left). The rate of phosphorus uptake in natural water is again seen to e much greater than in any of the artificial solutions.

Another factor which was thought worthy of investigation was the effect of H on the rate of phosphate uptake of depleted cells. A series of identical spensions of *Asterionella* cells in Windermere North Basin water was set up. he pH of the series was varied by addition of N/1000 HCl, and 25 μg.P/l. as added to each flask. The pH and the phosphate remaining in solution fter a period of 2 hours standing in the light was measured in each case. esults are given in Table III.

It is seen that the uptake rate for phosphate is markedly reduced by lowering he pH, and this effect was investigated further by producing a similar series f cell suspensions in which the pH was extended both above and below eutrality by the addition of potassium carbonate and hydrochloric acid espectively. In this experiment 500-ml. suspensions of cells in lake water ere used, the pH being adjusted by addition of N/100 K_2CO_3 solution and 100 HCl, and measured in each case electrometrically. 50 μg.P/l. was added each flask and the phosphate remaining in solution after various periods as measured. The results are given in Fig. 9.

TABLE III

pH	P conc. remaining after 2 hrs.
	µg.P l.
6·8	1·0
6·5	1·0
6·3	1·0
6·1	2·0
5·9	5·0
5·5	10·0
5·3	17·5

That pH variation is by no means the only controlling factor in the rate phosphate uptake is demonstrated by the following experiment, in which th pH of distilled water is made equivalent to that of lake water. It is seen th pH variation in distilled water does modify the rate of phosphate uptake this medium, but does not make the rates equivalent for distilled water ar natural lake water.

TABLE IV

P concentration in µg.P l. remaining after times indicated

Each suspension contained 20,000 cells ml.

Treatment	pH	0·5 hrs.	1·5 hrs.	2·5 hrs.	3·5 hrs.	4·5 hrs.	5·5 hrs.	21·5
Windermere water + 200 µg.P/l.	[illegible]9	192	176	164	152	144	140	72
As above + 50 µg.P/l.	6·9	37	29	22	15	9	4	4
Distilled water − K_2CO_3 − 50 µg.P l.	6·8	47·5	45	42·5	30	36	36	18
Distilled water + 50 µg.P/l.	5·9	48·5	47·5	47·5	48	45	43	40

Equivalence of both total ionic concentration and pH between artific solutions and lake water might produce equivalent behaviour in cells su pended in such media, but this is shown not to be the case by the ne experiment (see Table V).

TABLE V

µg.P/l. remaining after times indicated

Flasks contained initially cells + 50 µg. P l. and solutions indicated

Time (hours)	Windermere water	M 1,000 $Ca(HCO_3)_2$ aerated till pH became 7·4	M 1,000 KC pH 7·1
0·5	35	46	46
1·0	23	46	44
2·0	11	40	39
18·0	2	30	28

The foregoing experiments throw little light on the puzzling behaviou cells suspended in artificial solutions as compared with those suspended lake water, and it can only be concluded that the lake waters contain some fac which is not simply imitated by synthetic solutions.

As a further step an experiment was carried out by treating lake water in rious ways which might suggest the nature of the unknown factor.

TABLE VI

Identical samples of Windermere North Basin water were treated in the following ays; to each sample was added the same amount of cell suspension + 50 μg.P/l. osphate remaining after 1 and 2 hours is given in μg.P/l.

Treatment	P after 1 hr.	P after 2 hrs
ake water evaporated to dryness on a hot plate and made up to original volume with distilled water .	50	45
ake water evaporated to one-fifth of its original volume and rediluted with distilled water to original volume	40	32
esidue after evaporation heated just to charring point and then redissolved in distilled water to the original volume (pH = 6·5)	43	31
harred residue from lake water similar to (3) but dissolved in hydrochloric acid and evaporated to remove excess acid, then diluted to original volume with distilled water (pH = 6·5)	40	30
ake water control untreated	27	17
istilled water	42·5	45

Here again the fastest uptake of phosphorus takes place in the untreated ater; partial evaporation of the water reduces the rate of phosphorus uptake o an extent comparable with charring the lake water residue and re-solution.

It is hoped to continue this investigation further in an attempt to find the ature of the factors which influence the rate of phosphorus uptake of de-leted cells of *Asterionella*.

DISCUSSION OF LABORATORY AND FIELD OBSERVATIONS

It seems clear that the minimum amount of phosphorus required by *Asterionella* is very small indeed, so small in fact that an initial concentration of 1 μg.P/l. would supply the needs of any normal growth of this diatom in Windermere.

The cells, however, have a marked ability to accumulate phosphorus even from very low external concentrations, so that in Windermere when growth is slow in winter the few cells present can maintain a reserve of phosphorus which may be used later for cell division even during a scarcity of phosphorus, with a consequent fall in cell phosphorus. In media of high phosphorus concentration the cell phosphorus content does not fall during cell division, so that depletion of phosphorus takes place in the medium and not in the cell. This means that the growth of cells in the presence of abundant phosphorus leads to large-scale depletion of the medium, as is observed in natural waters having high initial phosphate concentrations (Gardiner, 1941), while in waters of low initial phosphate concentration the depletion takes place in the cell phosphorus accumulation, and no large fluctuation is observed or is indeed possible in the water itself.

It seems that in the case of Windermere it is unnecessary to postulat utilization of either 'organic' or adsorbed phosphorus as immediate sources o supply additional to the dissolved phosphate, though these reservoirs ma obviously play a part in the long-term replenishment of phosphate ion by th breakdown of 'organic' phosphorus, which cannot in any case be used directl by the cells, and the de-sorption of phosphate ion from ferric hydroxide either in suspension or in the mud, to maintain equilibrium with the phosphat in solution.

It is possible to postulate a sequence of events in the production of larg numbers of *Asterionella* cells in Windermere on the following lines.

In the winter period, when division is slow due to low light intensity, th replacement rate of phosphate ions is such that all the cells can maintain high phosphorus content without removing phosphorus faster than it is re placed. In this way the population which serves as an inoculum for the sprin outburst is rich in phosphorus. When the light increases in intensity, divisio takes place at a faster rate, until the cell requirement for phosphate outstrip the rate of replenishment; at this time the cell phosphorus begins to fall. Th fall in cell phosphorus continues, supplying new cells with phosphoru possibly without reference to the very low phosphate concentration in th water, until other factors (normally lack of silica) eventually cause growth t cease, before the cell phosphorus has fallen to a dangerous level.

In waters rich in phosphorus the mechanism would be slightly differen the starting cells are rich in phosphorus as before, but as more cells are produced, phosphorus is available in abundance to enable even larger populatio to maintain a high cell phosphorus; thus the phosphorus is removed from th water with little depletion in the cells, producing a marked correlation betwee fall in phosphorus concentration in the water and growth of the cell population. Apparently, then, analysis of the water can be of little assistance i determining whether or not phosphorus deficiency is limiting growth; on th other hand, analysis of the cells may give valuable information.

Fluctuations in the amount of phosphorus contained by the cell are n confined to *Asterionella formosa*, as evidenced by the work of Ketchum ar Redfield (1949) on *Chlorella pyrenoidosa*. These authors found that th phosphorus content of *Chlorella* was reduced by growth in media poor in th element, and that the 'phosphorus debt' so induced could be made good if th depleted cells were immersed in a medium containing phosphorus either the light or dark. This behaviour of *Chlorella* closely parallels that found *Asterionella*.

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Microbiological Synthesis of Fat

THE EFFECTS OF VARYING PH, TEMPERATURE, NITROGEN, INCUBATION PERIOD, AND SUGAR CONCENTRATION ON FAT-PRODUCING MOULDS

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SUMMARY

1. A more detailed study has been made of the influence of these factors on fat formation by *Aspergillus nidulans*, *Penicillium spinulosum*, and *Penicillium javanicum*.

2. The effect of halving the glucose, while keeping the ammonium nitrate concentration constant, lowered the yield of fat on sugar used in *A. nidulans* and *P. spinulosum* but not in *P. javanicum* cultures.

3. Keeping the same N:C ratio and raising the glucose concentration from 10 to 20 per cent. showed that 10 per cent. glucose was more efficiently converted to fat by *A. nidulans* and *P. javanicum*.

4. The iodine values of the extracted fats were higher, in general, with increased length of incubation. Low ammonium nitrate concentrations, however, tended to give low iodine values.

5. The results have been applied on a larger scale by growth in Roux bottles, Glaxo flasks, and a flat stainless-steel tank.

THE possible applications of mass culture of micro-organisms in relievir prospective nutritional deficiencies in the world's food-supply is receivir increased attention. In a preliminary survey (Woodbine, Gregory, and Walke 1951) some of the more interesting moulds regarded as potential fa producers were presented. Apart from the *Mucor* and *Fusaria* spp., whic have been the province of German and United States research, some seven eight strains were of interest.

Three of these, *Aspergillus nidulans*, *Penicillium spinulosum*, and *P. javanicu* have been examined in greater detail. In the present study the moulds ha been grown on one medium only (see Prill, Wenck, and Peterson, 193 Woodbine *et al.*, 1951). As the N:C ratio is an important factor in fat productic (Bichkovskaya, 1940; Heide, 1939; Bernhauer, Niethammer, and Rauc 1948), different sugar and ammonium nitrate concentrations have been use The influence of two incubation temperatures, 25 and 30°, has been examin also (see Pearson and Raper, 1927). Although Lockwood, Ward, May, Herric and O'Neill (1934) did not consider the initial pH of the medium to be critic the moulds have been grown at a pH of 4·8 and 6·8. In addition, incubati periods of 7, 10, and 14 days have been used and the iodine values of some

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e extracted fats estimated. The initial experiments were performed using 5-ml. media and later extended to larger containers of different surface to olume ratio.

EXPERIMENTAL

Strains. Aspergillus nidulans Eidam, *Penicillium javanicum* v. Beyma, and *enicillium spinulosum* were the same as those used previously (Woodbine *et l.*, 1951) and were similarly maintained.

Media. The basic chemically defined medium was that of Prill, Wenck nd Peterson (1935). Sterilization was by autoclaving for 15 minutes at 10-lb. ressure.

Inoculation and harvesting. The 100-ml. Erlenmeyer flasks contained 25-ml. nedium and each series was inoculated with a spore+mycelium suspension of ne of the three moulds. The flasks, five from each series, were incubated at 5° and at 30° for 14 days.

The harvesting, drying, grinding, fat extraction, and estimation of sugar vas as before (Woodbine *et al.*, 1951).

Iodine values. These were obtained by Yasuda's (1931) modification of the riginal method of Rosenmund and Kuhnhenn (1923) using a prepared eagent (B.D.H.).

RESULTS

Four series of experiments were run and a large mass of detailed analytical data obtained (see Gregory, 1950; Woodbine, 1951) which can only be quoted in a summarized form here.

Series I. The three moulds were grown in small flasks on 20 per cent. glucose with NH_4NO_3, the optimum concentration of which was found to be 0·35 to 0·25 per cent. Fat yields varied from 2·3 to 5·9 g. per 100 g. sugar used or 10–16 g. per 100 g. mycelium.

Series II and III. The glucose concentrations were reduced to 10 per cent. and NH_4NO_3 concentrations varied from 0·5 to 0·175 per cent. It was hoped that it would be possible to decide whether 10 or 20 per cent. glucose was optimal when the N:C ratio was the same at the two sugar concentrations. Harvesting in series III consisted of five flasks taken after 7, 10, and 14 days incubation. The maximum weight of fat was obtained after 10 or 14 days. Since fat was extracted with light petroleum, it is possible that the quantities may be underestimated and that extraction with chloroform, ethanol, or the HCl treatment suggested by Nägeli and Loew (1878) and Smedley-McLean (1922) would have given higher yields. The results are summarized in much abridged form in Table I.

Series IV. Larger units were used. Inoculations were made from 25 ml. in Erlenmeyer flasks incubated for 2 days. Vessels used were, for each fungus: 6 Roux bottles containing 150 ml. culture fluid+inoculum; 3 Glaxo flasks containing 200 ml.+inoculum; 7×100 ml. Erlenmeyer flasks containing 25 ml., and 1×1,000 ml. stainless-steel tank.

The results are summarized in Table II.

TABLE I

Summarizes the effects of temperature, glucose concentration, and N:C ratios o fat production

Mould	Glucose (%)	NH_4NO_3 (%)	Temp. (° C.)	Fat coefficient (g./100 g. sugar utilized)
A. nidulans	20	0·50	25	5·3
	10	0·25	25	4·5
	20	0·35	25	4·7
	10	0·175	25	10·0
	20	0·50	30	2·7
	10	0·25	30	4·1
	20	0·35	30	4·4
	10	0·175	30	4·9
P. spinulosum	20	0·35	30	2·3
		0·25	30	2·6
	10	0·15	30	1·4
P. javanicum	20	0·35	30	5·9
	10	0·175	30	7·2

TABLE II

Mycelial weights in g. (M) and fat yields in g. per 100 g. sugar utilized (F and in g. per 100 g. mycelium (F/M) at pH 6·7

Mould	Container	Glucose 20% NH_4NO_3 0·5%			Glucose 10% NH_4NO_3 0·5%			Glucose 10% NH_4NO_3 0·25%		
		M	F/S	F/M	M	F/S	F/M	M	F/S	F/M
A. nidulans (25°)	Roux	11·6 (±0·37)	7·4	20·8	6·31 (±0·32)	2·6	7·04	6·52 (±0·19)	7·3 (±0·3)	19·1 (±0·6
	Glaxo	13·7	7·8	23·3	7·87	2·8	7·70	7·79	8·5	24·1
	Flasks	1·53 (±0·07)	5·5	17·6	0·81 (±0·06)	1·7	5·20	0·83 (±0·02)	7·9	22·9
	Tank	—	—	—	—	—	—	33·4	8·7	25·2
P. spinulosum (30°)	Roux	5·05 (±1·02)	1·2	7·56	5·15 (±0·42)	1·7 (±0·3)	5·83 (±1·31)	1·43 (±0·91)	0·6	5·82
	Glaxo	4·81	1·0	7·88	5·17	1·4	5·73	1·80	0·5	5·20
	Flasks	0·77 (±0·04)	1·8	9·46	0·71 (±0·03)	1·5	5·26	0·43 (±0·10)	1·0	5·03
	Tank	—	—	—	12·3	0·75	5·90	—	—	—
P. javanicum (30°)	Roux	11·5 (±0·48)	4·9	14·5	5·45 (±1·07)	3·9 (±1·0)	11·3 (±4·67)	5·58 (±1·62)	5·7 (±2·4)	16·7 (±4·6
	Glaxo	14·1	4·2	13·1	7·62	4·1	12·0	7·84	7·1	19·9
	Flasks	1·58	4·0	12·7	0·88 (±0·04)	3·4	10·5	0·91 (±0·11)	5·7	15·4
	Tank	32·3	1·6	7·92	—	—	—	—	—	—

DISCUSSION

The difficulties in reaching conclusions on a basis of the shifting sands biological variability are now well known. In this work on fat-producin moulds *P. javanicum* has exhibited such a variability to a high degree, a resu not unexpected in view of the results and comments of Lockwood *et al.* (193

Although the physical conditions operating on the growing moulds a important, the chemical effects of sugar concentration and the continge N:C ratio limit the rate and quantity of fat production, see also Heide, 193 rnhauer, 1943; Raveux, 1948; and Hesse, 1949. A concise summary of this neral thesis might well be that providing the nitrogen and carbon levels are ch as to provide adequate growth and a suitable matrix for the laying down fat, or protein, then a high N:C ratio favours protein and a low N:C ratio vours fat production—both being on a quantitative basis.

In contrast to the results of Pearson and Raper (1927), we did not find that e lower temperature of 25° gave a fat extract with a higher iodine value. heir suggestion was based on the association of a lower melting-point with eater unsaturation. This point needs further development in view of the ndamental importance of the unsaturated fatty acids as essential growth ctors which has become apparent in recent years. We did find, however, at greater unsaturation was associated with longer incubation. The general nclusion from this previously would have been that the longer period abled synthesis of the unsaturated acid(s) to proceed from the already nthesized saturated fatty acids. This view has been opposed recently by ilditch (1951), who has suggested that syntheses of both types proceed parately, but possibly from a common precursor. This whole subject of tty acid production, however, can only be satisfactorily evaluated on a basis f quantitative estimations of particular and individual fatty acids (Lovern, 952). The complications engendered by mixtures of the fatty acids themselves gether with attendant phospholipids and lipoproteins vitiates the possibility f drawing firm conclusions on the basis of iodine value determinations.

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Further Observations with the Electron Microscope on Spermatozoids in the Brown Algae

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SUMMARY

Observations are recorded for *Ascophyllum*, *Pelvetia*, *Himanthalia*, and *Dictyota* in addition to *Fucus* which has already been described in a previous paper. Fibrillar disintegration of cilia has been obtained in all except *Pelvetia*, in each case to give nine peripheral strands and a central pair. This corrects a previous error for the hind flagellum of *Fucus*. Some facts are given regarding the internal organs associated with the parts of the cilia inside the body in *Himanthalia* and *Dictyota*. A proboscis similar to that previously described for *Fucus* has been demonstrated in *Ascophyllum* and *Pelvetia*, but is absent from *Himanthalia* and *Dictyota*. *Himanthalia* differs from the other Fucoids in the relative lengths of front and hind flagella. *Dictyota* has only a single flagellum. In all, the front flagellum is a *Flimmergeissel* with two rows of hairs, which, in certain cases, notably *Himanthalia*, have very long hair points. In *Himanthalia* there is a large spine near the distal end of the front flagellum borne on one fibril of the peripheral series. In *Dictyota* there is a row of smaller spines at the front end of the flagellum borne in a line between the two lateral rows of hairs. These spines in *Dictyota* can be used as evidence regarding the internal symmetry of the whole cilium which is summarized in a diagram.

INTRODUCTION

THE new work on Brown Algae to be described below has consisted on the ne hand in applying the staining technique recently devised by A. D. Greenwood and described in Manton, Clarke, Greenwood, and Flint, 1952, to some f the more refractory cilia, notably the hind flagella in the Fucoids, with he object of perfecting the observations on their fibrillar composition. On the ther hand, we wished to ascertain the distribution, among other genera, of the ery curious membranous proboscis described in the spermatozoid of *Fucus erratus* (Manton and Clarke, 1950, 1951*b*), in order to get some insight into ts possible function. Both methods of approach have met with a considerable degree of success. The fibrillar structure has been completely ascertained in *Ascophyllum*, *Fucus*, *Himanthalia*, and *Dictyota* and shown to conform exactly o the prevailing type in all other plant cilia, thereby removing an apparent anomaly. In addition some wholly unexpected new facts have been revealed n the last two genera which are not only of interest in themselves in a number of different ways but also contribute to our knowledge of the internal structure of cilia in general.

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For the convenience of the reader who may not be familiar with the published accounts of the spermatozoid of *Fucus* (loc. cit.) we propose to dea with the five genera in arbitrary order starting with *Ascophyllum*. This is s like *Fucus* in almost every particular that it will serve to recall the genera structure found in *Fucus*. Somewhat unexpectedly, *Himanthalia*, although a member of the Fucoid affinity, offers almost as striking a contrast as doe *Dictyota*.

ASCOPHYLLUM

Fig. 1, Pl. I, shows a general view, at our standard low-power magnification of × 3,000 diameters, of a zoospore of *Ascophyllum nodosum*. It differs from *Fucus serratus* only in having a slightly larger body with somewhat shorte cilia and a markedly shorter hind flagellum compared with the front flagellum The relative dimensions of these two organs are approximately 3:1 in *F* *serratus* but only about 2:1 in *Ascophyllum*. Nevertheless, in both the hind flagellum is undoubtedly the longer, a feature which hitherto has been though of as characteristic of the Fucaceae (cf. Fritsch, 1945, vol. 2), in contrast t other members of the brown algae in most of which (e.g. *Pylaiella* an *Laminaria*, Manton and Clarke, 1951*a*) the hind flagellum is very short compared with the front one.

In *Ascophyllum*, as in *Fucus*, the front flagellum is a *Flimmergeissel* although the hairs break off rather readily and only their bases are still visible in th more highly magnified specimen of Fig. 2, Pl. I. Since *Ascophyllum*, unlik *Fucus*, can apparently not be hardened in formalin we have not attempted t study the *Flimmer* hairs for this genus in greater detail. The proboscis is however, easy to demonstrate. It can be seen clearly both in life and on th low-power view of Fig. 1, Pl. I, while the details of its concentric banding ar beautifully revealed in Fig. 2, Pl. I. There are of the order of 14–15 bands a opposed to the 13 found in *F. serratus*, a numerical difference which seems t be correlated with the somewhat larger size of the organ in *Ascophyllum* Further, the evidence from fibrillar disintegration (Fig. 3, Pl. II), which occurs far more readily in this genus than in *Fucus*, indicates that the opaqu bands on the proboscis, which were illustrated without further description i *Fucus* (Manton and Clarke, 1951*b*), are in fact fibrous thickenings somewha resembling in dimensions the fibrillar components of cilia. They are shown in the dismembered state in the upper part of the field in Fig. 3, Pl. II. In th intact organ the fibrils are gathered together at the base of the proboscis an pass along the body as a coherent band. This band appeared structureless i *Fucus* (Manton and Clarke, 1951*b*, Fig. 15) but it is clearly fibrillar in *Ascophyllum* (cf. right lower side of the specimen of Fig. 2, Pl. I).

Fig. 3, Pl. II, gives the facts for fibrillar disintegration in *Ascophyllum* from a stained specimen selected with visual light and stripped from glass. In addition to the dismembered proboscis it shows very clearly the fibrillar composition of both flagella, which can easily be distinguished from each other b their relative length and by the presence of *Flimmer* hairs still detectable o e front one. In both flagella the internal construction is the same. There are well marked central pair of strands and nine peripheral ones.

FUCUS

The only point of interest in this genus in the present connexion concerns e fibrillar composition of the hind flagellum of *Fucus serratus*, and we have onfined our attention to that. In our previous account (Manton and Clarke 951*b*) we had illustrated two specimens of the front flagellum, in each of hich eleven strands were visible, but even after prolonged search we were nly able to find a single imperfect specimen of a dismembered hind flagellum, which we could detect only nine strands. We attached no significance to this the time since we had felt that the two flagella might reasonably be expected differ in their internal construction as well as in external morphology. It as only later, after we had examined ferns, mosses, green, yellow-green, and rown algae of other genera, and fungi (cf. postscript to Manton, 1951), in l of which only the number eleven had been found, that we realized the nportance of ascertaining whether or not the anomalous record for the hind agellum of *Fucus* might in fact be an error. To test this it was essential to udy more specimens, and to obtain these it was necessary to stain a large umber of preparations of cells dried down on glass after killing with osmic apour in the usual way, in order to detect the very infrequent cases showing smemberment of the right kind. Fibrillar disintegration, especially of the ind flagellum, is extremely rare in *Fucus serratus*, but when it does occur, the easons for our previous record are at once apparent. The nine peripheral rands are relatively resistant to further decomposition but the central pair not. In specimens such as those of Figs. 4, 5, and 6, Pl. III, the central rands are present but they are very faint, and had we not been looking for em attentively they might have escaped observation. We therefore conclude at our previous record was an error based on a specimen in which the central air of strands had decomposed, and that except for this mechanical weakness e hind flagellum in *Fucus* differs in no essential way from the other types of lia met with in plants.

PELVETIA

The very common British species *Pelvetia canaliculata* has the great disdvantage for electron microscopy that the conceptacles are bisexual and erefore both types of gamete are exuded together. This virtually precludes e making of direct preparations of living cells straight onto formvar carriers nce the eggs are so large that they would immediately endanger the stability f the film on entering the microscope.

In order to separate the spermatozoids from the eggs the mixed suspension as therefore killed on glass slides with osmic vapour, then dried, rinsed, and ried again. Suitable fields containing spermatozoids but no eggs were then ripped off and remounted for examination with the electron microscope.

Their condition was found to be distinctly unfavourable to detailed study but
they are perhaps of interest for comparative purposes.

The low-power view of Fig. 7, Pl. III, shows a spermatozoid of the *Fucus*
type but smaller. Fig. 8, Pl. III, shows the proboscis which is also of the *Fucu*
type but smaller, containing only eleven bands of thickening instead of the
thirteen of *Fucus* or the fourteen to fifteen of *Ascophyllum*. This is the only
observation we have attempted to make on this material, but we quote i
because the numerical variation of these bands with the size of the organ
itself is in striking contrast to the extreme constancy of the fibrillar number in
cilia of all sizes.

HIMANTHALIA

Spermatozoids of *H. lorea* were obtained in great abundance from the coas
of Anglesey, near Rhosneigr, at the spring tides in the first week of Septembe
1952. Adult plants were collected at low tide with the assistance of Dr. Marti
of the Botany Department of the University College of North Wales, to whom
we are most grateful for invaluable assistance. Male and female plants were
separated by microscopic examination on return to the laboratory, and lef
overnight in two large glass jars kept covered so that the plants remaine
moist but not wet. Next morning the gametes had oozed copiously out o
every conceptacle, and the orange ooze from the male conceptacles liberate
very actively swimming spermatozoids in a few seconds after being put bac
into sea water.

This material was treated in three ways. A number of direct preparation
were made at once onto previously prepared formvar carriers in the usual way
by killing with osmic vapour followed by drying, rinsing, and again drying
These gave most of the morphological pictures and all the dismemberment
Other similar preparations were made onto glass slides with a view to strippin
later. This was done but the result was inferior to the direct preparation
Finally, residual material was killed in bulk with osmic vapour and preserve
in formalin diluted with sea water to give an ultimate strength of 4 per cen
Our experience with *Fucus* had shown that extra hardening with formalin ca
be very beneficial for the preservation of the details of the hairy appendage
and this was also the case here. In the direct preparations the *Flimmer* hai
break off near their bases (cf. Fig. 12, Pl. V) as in *Ascophyllum*, but prepar
tions made from the formalin hardened material gave beautiful detail of th
hairy appendages (Fig. 13, Pl. V), although these are so long and lax that th
inevitably become somewhat dishevelled with so much handling. We ha
therefore found it convenient to combine results from both types of prepar
tion in presenting the facts.

The final dehiscence after immersion in sea water of the antheridia outsi
the conceptacle is very much as in other members of the Fucaceae. The a
theridia themselves are rather large, but their empty skins remain in the su
pension among the swimming spermatozoids and can easily be picked
(Fig. 9, Pl. IV). This can also be done with *Fucus* and *Ascophyllum*, and

ave pictures of both which we have not so far had opportunity to publish. *ucus* differs from *Himanthalia* in that the inner antheridial wall breaks down t both ends leaving a barrel-shaped empty skin, while *Ascophyllum* differs in aving rather smaller sporangia, opening at one end but more nearly spherical n shape.

The motile spermatozoids are nevertheless surprisingly unlike those of the ther Fucoids which we have studied. Two intact cells and a dismembered ell are represented in Fig. 10, Pl. IV. The first difference from *Fucus* is in the elative lengths of the two cilia, the hind one in *Himanthalia* being short with whiplash point as in the zoospores of the majority of brown algae (cf. ritsch, 1945). There is no proboscis but the front flagellum is a *Flimmer-eissel* with some new features which we have not previously encountered. The most conspicuous of these is a large hook or spine projecting laterally rom the flagellum near the distal end. The *Flimmer* hairs stop at this point, he distal end of the flagellum being free from them (Fig. 11, Pl. V), but it is o narrower than the flagellum axis in the hairy region and it does not there-ore resemble the ordinary type of 'whiplash' which we have encountered lsewhere, notably on hind flagella. The hairs, where present, are in the usual wo ranks, cf. Fig. 12, Pl. V, and where they have remained intact, i.e. in ormalin hardened material (Fig. 13, Pl. V), they can be seen to be long and with very conspicuous hair points displayed even more prominently than in he other types in which we have recorded hair points, notably *Ochromonas* Manton, 1951), *Pylaiella* (Manton and Clarke, 1951*a*), and *Saprolegnia* Manton, Clarke, and Greenwood, 1951).

Fibrillar disintegration was encountered repeatedly in the direct preparations. Fig. 14, Pl. VI, shows an early stage which is important as a demonstration of the existence of a skin outside the fibrils in both parts of the flagellum. Fig. 15, Pl. VI, shows the distal end of a completely dismembered front flagellum showing the usual eleven strands with the central pair if anything somewhat longer than the other nine, an arrangement which we have not usually found. The *Flimmer* hairs lie loose upon the field near their original points of attachment, but this lateral spine is much more resistant than the other parts of the flagellum. It is still intact and still in contact with its base which can be seen to be carried by a single fibril of the peripheral series. Once they have been recognized, it is easy to pick out numerous cases of detached spines lying loose upon the field as the last remains of completely disintegrated cells. Wherever disintegration is less complete, however (cf. for another example, the disintegrated cell in Fig. 10, Pl. IV), the spine can be seen to be borne by a single fibril.

Fibrillar disintegration of the hind flagellum is also easily obtained and shows the usual eleven strands. The cell of Fig. 10, Pl. IV, gave the count very clearly though at the magnification reproduced it may not be clearly visible. Another example is in Fig. 16, Pl. VI. This, however, has been inserted less for the numerical demonstration of the fibrils of the two cilia than for the information it gives about the mutual attachments of the two cilia to each other and to the body. We have not previously been able to study this except very imperfectly in a few cells of *Fucus serratus* in which the body had become partially transparent (Manton and Clarke, 1951*b*). Much further work will be needed before we can interpret all the features now displayed. It is, however, at once interesting to note that there is a 'bulbous base' at any rate or the hind flagellum (right, in the picture) recalling those seen in the fung *Allomyces* and *Olpidium* (Manton, Clarke, Greenwood, and Flint, 1952) There are also 'roots' of various sizes, including a large one which is possibl double and certainly fibrillar, projecting out to the left in Fig. 16, Pl. VI, belo the base of the *Flimmergeissel*. This 'root' bears a superficial resemblance to reduced proboscis (cf. Fig. 3, Pl. II), though we are inclined to interpret it a an internal strand rather than as an external organ on our present evidence It requires further study.

DICTYOTA

D. dichotoma is the first example which we have attempted to study of markedly oogamous brown alga which is not a member of the Fucales. It breeding habits, and in particular the periodic liberation of gametes at th spring tides, have been known since the pioneer observations of Lloy Williams (1897–1905). Accordingly we chose the spring tides at the beginnin of September 1952 and collected material from Lloyd Williams's own localit in the Menai Straits.

We are deeply indebted for this part of the work to Dr. M. T. Martin an the staffs of the Marine Biological Station and the Botany Department c University College of North Wales, Bangor. Exploratory collections of variou sorts were tried out, but the material actually used for the electron microscop was dredged from a dinghy at half-tide on the day of the lowest spring tid from the region of the Menai Straits known as 'The Swillies' between the tw bridges connecting Anglesey with North Wales. This was Lloyd Williams' best locality, and it is certainly an unusually favourable one for the growth the species, to judge by the exceptionally large size and beautiful conditio of the thalli obtained. These grow in large tufts up to 6 in. in length, with ver clean and regular branching totally free from the rather contorted growt which is liable to affect plants in the littoral zone in other places. In the Men Straits they are permanently submerged and grow epiphytically on oth submerged algae, notably *Laminaria* and *Halidrys*.

Reproductive organs of all three kinds (antheridia, oogonia, and tetraspore were found in abundance, the three kinds of plants being easily separab with a pocket lens. A bundle of the male plants so isolated was closely wrapp in wet newspaper to prevent desiccation, since the thalli are much more de cate than in the Fucoids. In this condition they were left overnight in a cover dish. Next morning complete male plants were immersed in fresh sea wat in a watch-glass or petri dish and within a few minutes liberation of gamet began and continued until the water contained a swarming population swimming spermatozoids. Some of these were then mounted directly previously prepared carriers in the usual way. They yielded the best results and only these have been effectively used. Similar preparations dried down on glass were less satisfactory, but formalin hardening proved to be completely useless since cells so treated disintegrated.

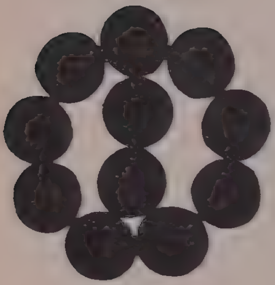
Fig. 17, Pl. VII, gives a low-power view of two cells of *Dictyota* with an unidentified other micro-organism lying between them. As described by Lloyd Williams, there is only a single flagellum, attached laterally. There is no proboscis.

Fig. 18, Pl. VII, gives a somewhat more enlarged view of two further cells, an intact one and a dismembered one; while still more highly magnified views of two additional complete cells appear in Figs. 19 and 20, Pl. VIII. From these various pictures it is obvious that we have again a *Flimmergeissel* of the usual type together with some novel features. The most important of these is the presence of a row of about twelve spines arranged in a line between the two rows of *Flimmer* hairs on the distal half of the flagellum. The spines do not obtrude themselves on the view when they are superposed on the flagellar axis as in Figs. 17, Pl. VII, and 19, Pl. VIII, though even here they can be seen on the original prints as dense patches across the axis. They are, however, very conspicuous when they are lying sideways and appear in silhouette Figs. 20, Pl. VIII, 21, Pl. IX). Since they lie between the rows of hairs they do not interrupt the continuity of the *Flimmer* which (Fig. 19, Pl. VIII) extends to the tip of the flagellum. Some further details in a more highly magnified part of a front flagellum can be seen in Fig. 21, Pl. IX.

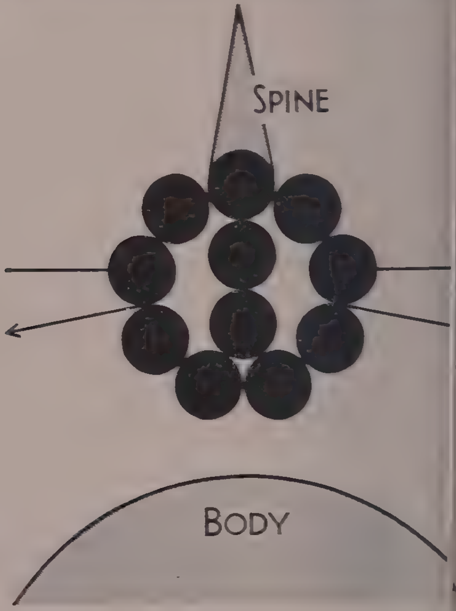
Several cases of fibrillar disintegration were obtained in the direct preparaions and they are very instructive. A complete specimen in this condition is represented in Fig. 18, Pl. VII, and the base of another is shown in Fig. 23, Pl. IX. That there are eleven strands in the flagellum, including a central pair, scarcely needs further comment. The base of the specimen of Fig. 23, Pl. IX, is, however, interesting. We were looking for signs of a second flagellum, and it is possible that the tail-like extremity is indeed a vestigial structure of this nature. Comparison with *Himanthalia* (Fig. 16, Pl. VI), however, indicates that one must be cautious in interpreting these internal structures until a larger number of cell types have been so studied. The presence of root-like extremities of several kinds in both species makes detailed interpretation difficult. The most hat can be hazarded for *Dictyota* on our present evidence is that a hind flagellum as an external organ is certainly absent, though it is not impossible that something homologous with it may still be represented as a vestigial structure inside the cell.

A more highly instructive detail concerns the attachment of the spines in dismembered specimens. Fig. 18, Pl. VII, shows one of these on a relatively small scale, while Fig. 22, Pl. IX, shows part of another much more highly magnified. The latter in particular will repay close study. The fibrils are somewhat broken and the full eleven can only be made out at the left hand end of the picture. Here, however, they are sufficiently continuous to make it quite certain that the spines form a row on one fibril only, and that this fibril is part of the peripheral series and placed in a median position between the two rows of lateral hairs.

This observation is of the first importance because it makes possible an almost exact correlation of the external features of the organ with its internal symmetry. The internal symmetry of the ciliary axis is indicated in a very diagrammatic way by Text-fig. 1, which is reproduced without change from



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Diagram to show the approximate distribution of fibrils in a theoretical org of cylindrical shape composed of nine peripheral strands and a central pair, all of equ diameter. Reproduced from Text-fig. 2*c* of Manton and Clarke, 1952.

TEXT-FIG. 2. The same, redrawn to include the known or probable positions of the vario appendages in relation both to the fibrils and to the cell body of the spermatozoid of *Dictyo* For further explanation see text.

Note: These diagrams are over-simplifications to express the facts of symmetry only. They are not compl reconstructions of true cilia since they omit components which are known to be there (e.g. sheaths and ski and also omit the known size differences between peripheral and central fibrils.

our paper on *Sphagnum* (Manton and Clarke, 1952), an organism in whi the cilia have no appendages. It is obvious from the geometry of the diagra that there is only one strand which could carry a median row of spines withc upsetting the bilateral symmetry of the organ, and this is the fibril plac uppermost in the diagram and which lies in the same vertical line as the cent pair. If the spines are indeed carried on this as indicated in Text-fig. 2 th there are only two likely places for the lateral rows of hairs. If these are a borne along single fibrils, they must occupy the positions marked by the t horizontal lines. If, however, the *Flimmer* hairs emerge between two fibr ie most likely place for them is indicated by arrows. We have not at present uite sufficient evidence to enable us to choose with certainty between these ilternatives, though the difficulty of finding attached hairs still in place after ie fibrils have all separated longitudinally gives a slight weight of probability) the position occupied by the arrows as being the correct one.

One further fact can be added. If a survey is made of a number of specimens, uch as those illustrated in Figs. 18, Pl. VII, 20, Pl. VIII, and 21, Pl. IX, in hich the teeth are lying in silhouette against the ground, it is found in the iajority of cases that the teeth, at least at the proximal end of the row, are icing away from the body of the organism when this is lying on its side. Vhere twists occur along the length of a cilium they are generally easy to etect as, for example, at the distal end of the flagellum in Fig. 20, Pl. VIII. n other cases, such as that of Fig. 18, Pl. VII, the continuity of the two rows of *'limmer* hairs is quite uninterrupted throughout the length of the cilium hereby giving positive evidence for the absence of twists. The general agreenent between the majority of such cases is a strong indication that the plane f bilateral symmetry of the cilium is at right-angles to the surface of the body nd that the isolated dorsal strand carrying the spines is placed outwards with espect to the body.

These are the principal new facts which have been supplied by *Dictyota.* n expressing them diagrammatically in the manner of Text-fig. 1 it will, of ourse, be realized that we have intentionally over-simplified the situation by mitting all other structural details except those under discussion. That some ther details could have been added, had we wished to do so, is nevertheless ndicated by places such as that marked by an arrow in Fig. 22, Pl. IX, in vhich further microstructure within individual fibrils is exposed. A fibril ppears to be composed of at least two microfibrils normally enclosed within common fibril sheath which has locally disappeared. This was also our conlusion in *Sphagnum* and our three-dimensional diagram for that organism ontains it. We have, however, preferred to use the simpler block diagram of Cext-fig. 1 as a basis for expressing our new observations, for the sake of larity in distinguishing conclusions which to us are not confirmatory but iew.

DISCUSSION

Although the new facts for *Dictyota* are probably the most interesting of hose contained in this paper, their interpretation is very closely dependent oth on the context and on previous knowledge of other organisms. This is rought out very clearly if we consider, for example, the biological implications of the spines. The different, but analogous, structure of *Himanthalia* suggests airly strongly that in both cases we are probably dealing with organs of imilar function, and that this function has something to do with facilitating ontact with the surface of the egg, although in neither case are there sufficient lirect observations of other kinds to substantiate this. By a similar process of easoning we may feel that the mere absence of a proboscis from the two genera carrying spines is perhaps an indication that the proboscis also is perhaps an organ of copulation, rather than a swimming or a sense organ (cf. Manton and Clarke, 1951*b*), although this again requires other types of observations to substantiate it.

An observation of a fundamentally different order of importance is, however, that of the fibrillar composition of cilia. Here the removal of the anomalous record for the hind flagellum of *Fucus* is the most significant new contribution, since we can now say that there are no exceptions yet detected to the generalization that in all the main plant groups (Bryophyta, Pteridophyta, fungi, green algae, brown algae, and yellow-green algae, cf. Manton, 1951) an identical structure has been revealed.

This structure is, moreover, not confined to plants. We have drawn attention on several previous occasions (cf. Manton, 1951) to cognate observations on animals, and as our present manuscript goes to press we have had the privilege of receiving from Dr. D. W. Fawcett[1] of Harvard Medical School some micrographs of his wonderful super-thin sections of cilia in a frog, a mollusc, and man. In each of these the sections show with dazzling clarity that there is a central pair of strands within a tube of nine strands in exactly the same type of spatial relation as in our own theoretical diagram reproduced as Text-fig. 1.

This agreement can be no accident and it has long been obvious (cf. Manton, 1951) that there must be a functional explanation of some fundamental kind for such a remarkably constant set of facts. Our evidence from *Sphagnum* (Manton and Clarke, 1952) enabled us to make certain suggestions about it. The new facts from *Dictyota* add others. As expressed in Text-fig. 2, a cilium is obviously a bilaterally symmetrical organ which is also dorsiventral and oriented in a regular manner. This is extremely clear where the axis is carrying no less than three separate lines of externally visible appendages. Exactly the same conclusion would, however, follow from the geometrical attributes of the number of strands, as expressed in Text-fig. 1, even in the complete absence of external appendages and even with a completely circular cross-section.

Since this conclusion thus applies to all the normal types of cilia yet investigated in both plant and animal kingdoms, we may perhaps legitimately regard it as probably the most fundamental contribution which the electron microscope has yet made to knowledge of ciliary microanatomy.

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Lloyd Williams, L. (1897). The antherozoids of *Dictyota* and *Taonia*. *Ann. Bot.* **11**, 545–53.

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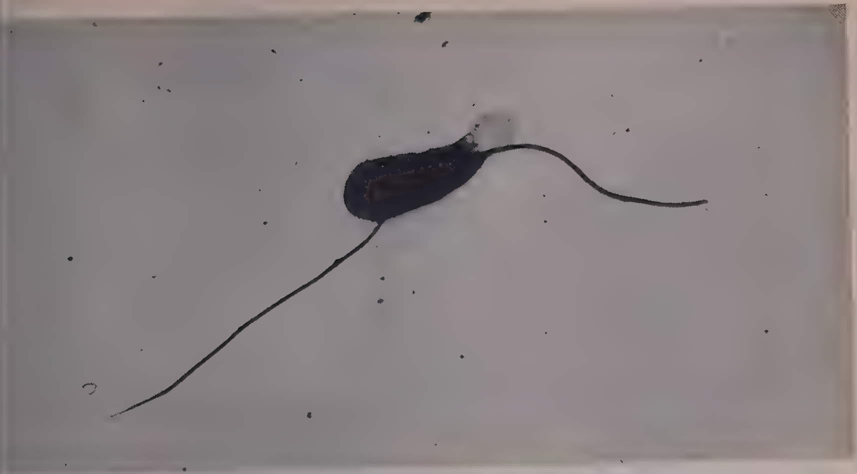
[1] We are very grateful to Dr. Fawcett for the personal communication of his results in advance of their formal publication, and for his permission to refer to them. We are also grateful to him for supplying the reference to Fawcett and Porter, 1952, in which a preliminary report (without illustrations) may be found.



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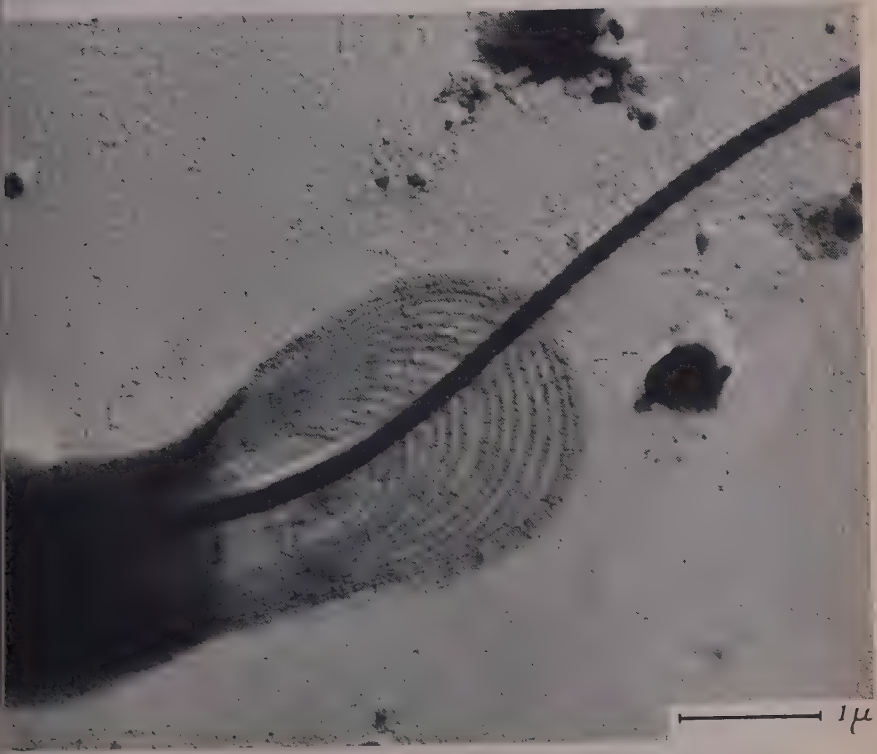

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FIG. 1. *Ascophyllum nodosum,* a spermatozoid. Electron micrograph M50·1, magnification ×3,000.

G. 2. More highly magnified part of another specimen to show details of the proboscis. Electron micrograph M95·21, magnification ×20,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 1

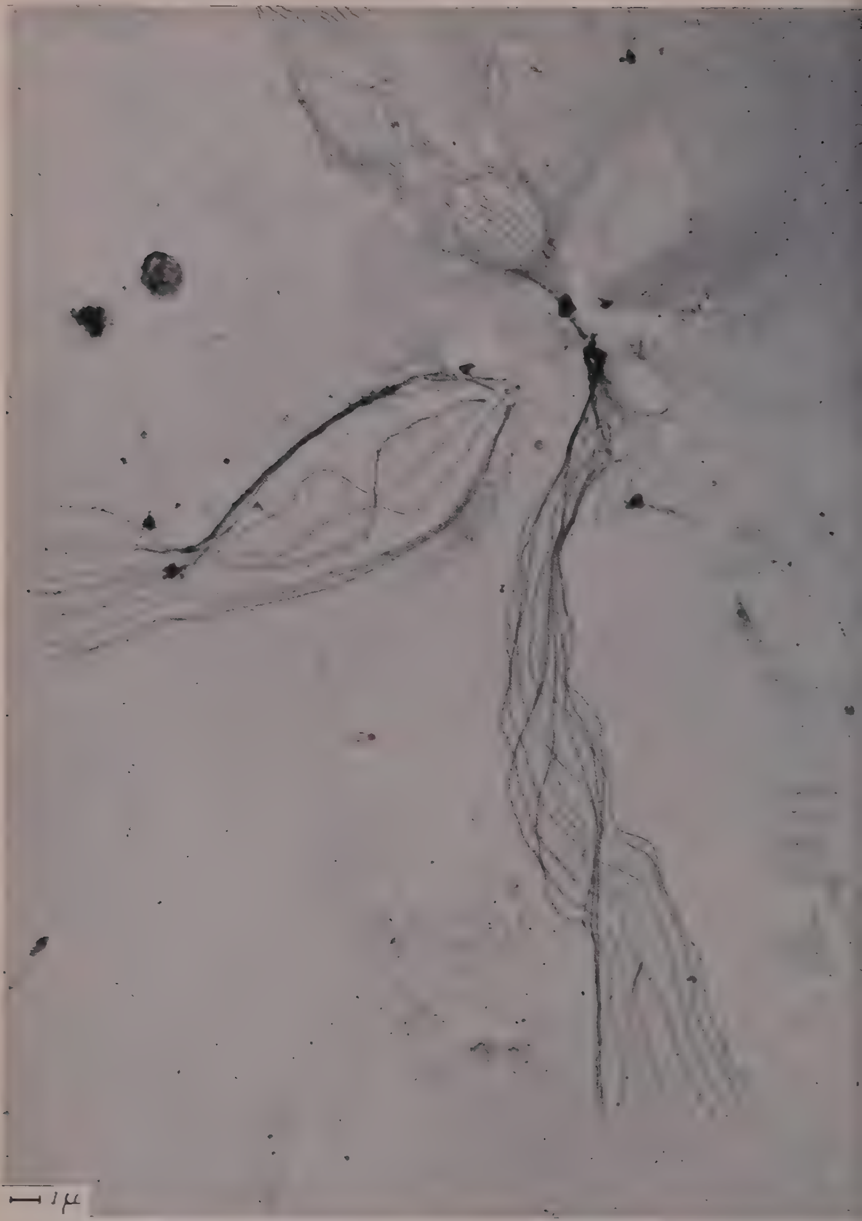
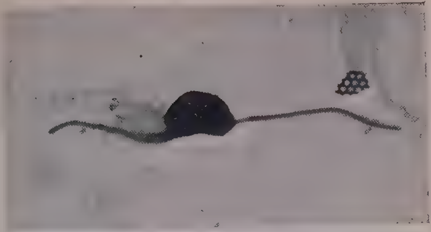


FIG. 3. *Ascophyllum nodosum*, a completely dismembered spermatozoid stained and stripped from glass. Electron n
graph M96·1, magnification ×4,500.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 2



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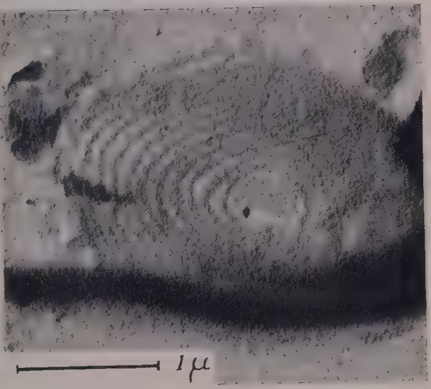

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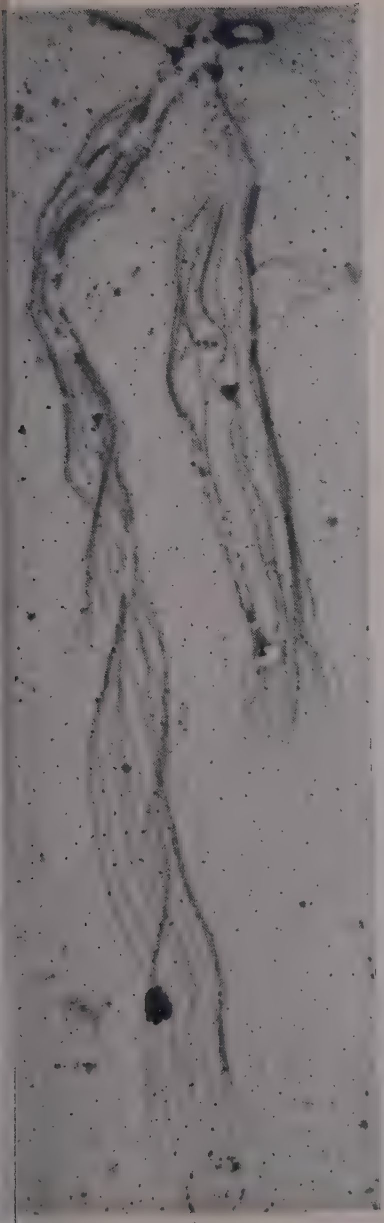

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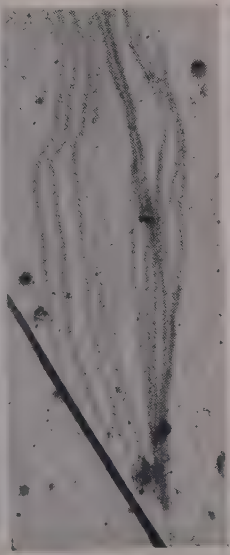

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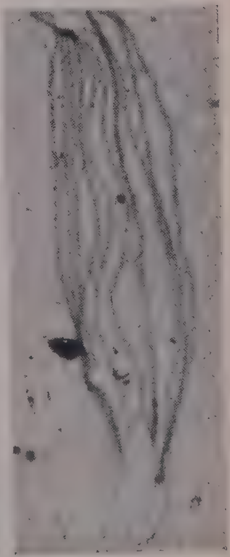

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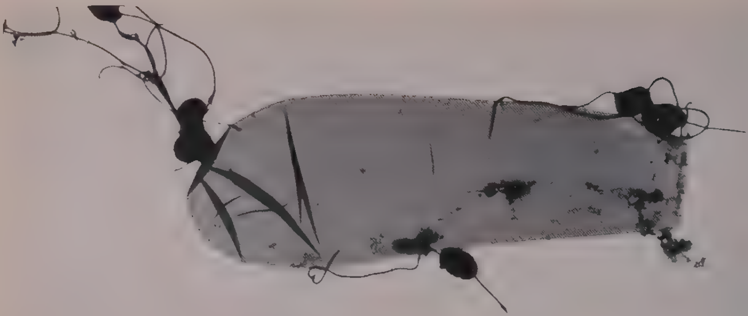
4. *Fucus serratus*, the two flagella of a dismembered spermatozoid stained and stripped from glass. Electron micrograph M97·3, magnification ×6,000.

5 and 6. Tips of hind flagella in two additional specimens otherwise like the preceding. Electron micrographs M90·8 and M89·14, magnification ×10,000.

FIG. 7. *Pelvetia canaliculata*, a spermatozoid. Electron micrograph M96·4, magnification ×3,000.

IG. 8. More highly magnified part of the same specimen. Electron micrograph M96·5, magnification ×20,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 3



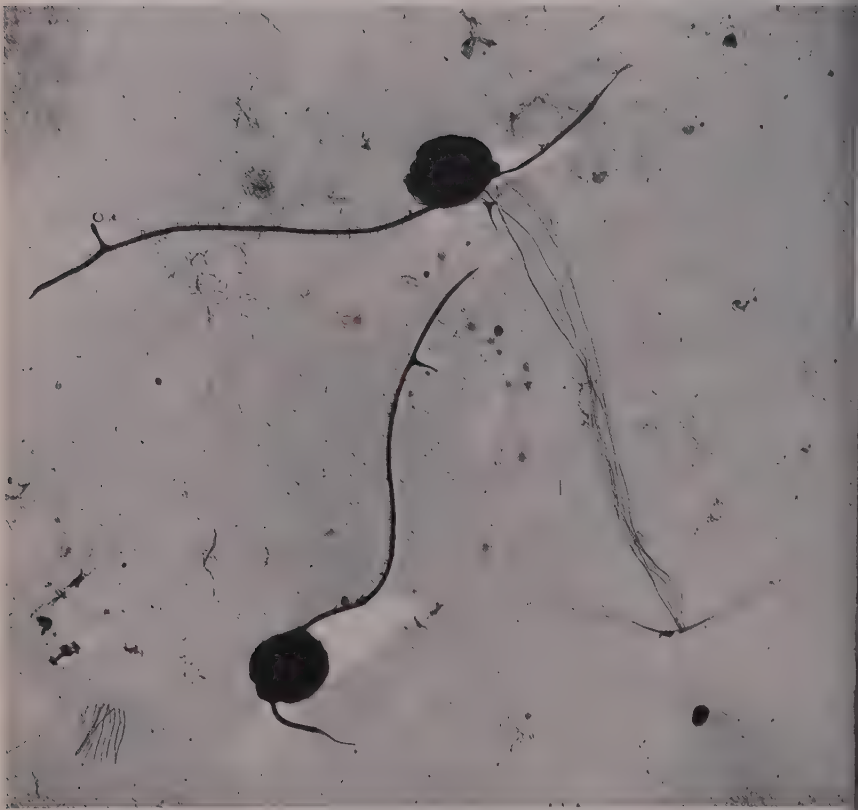
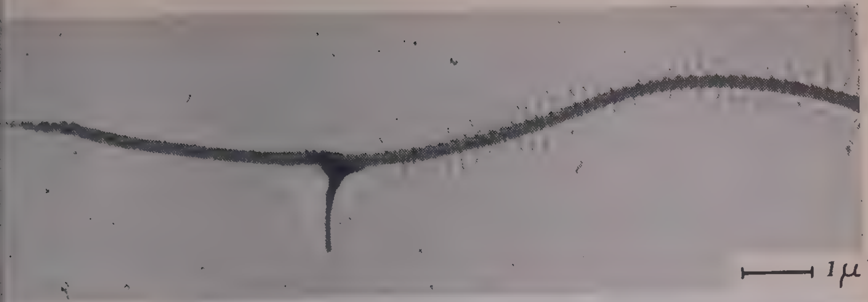


FIG. 9. *Himanthalia lorea*, the inner antheridial wall from which spermatozoids had recently been liberated. El micrograph M96·14, magnification × 2,000.

FIG. 10. Two intact and one dismembered spermatozoids of the same. Electron micrograph M92·17, magnification

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 4



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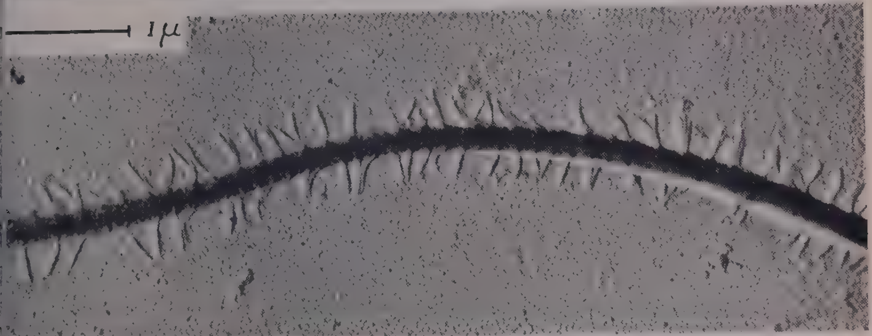

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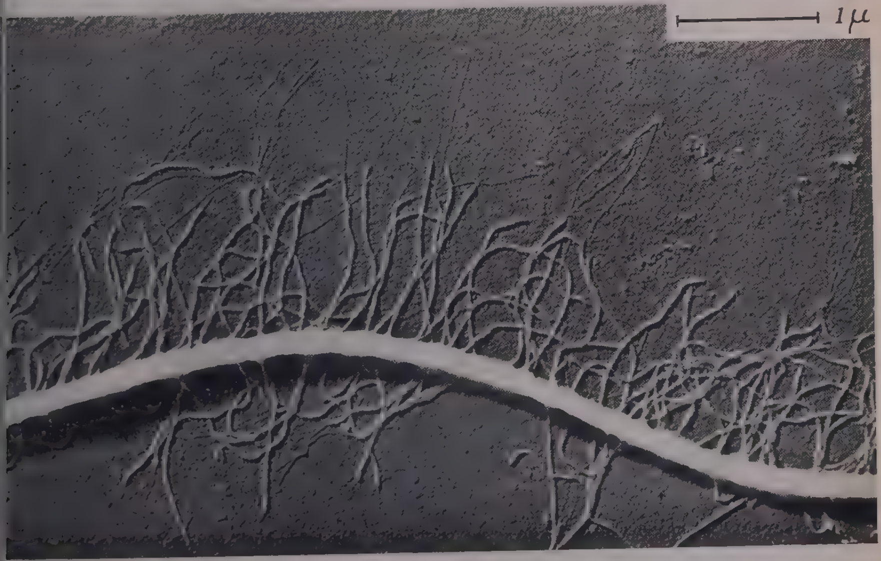

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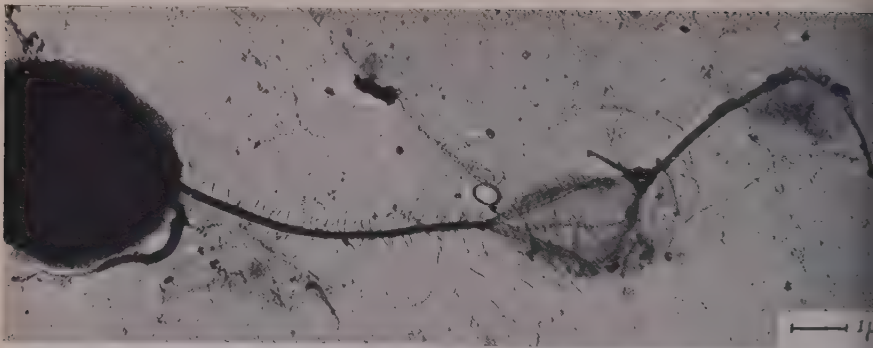
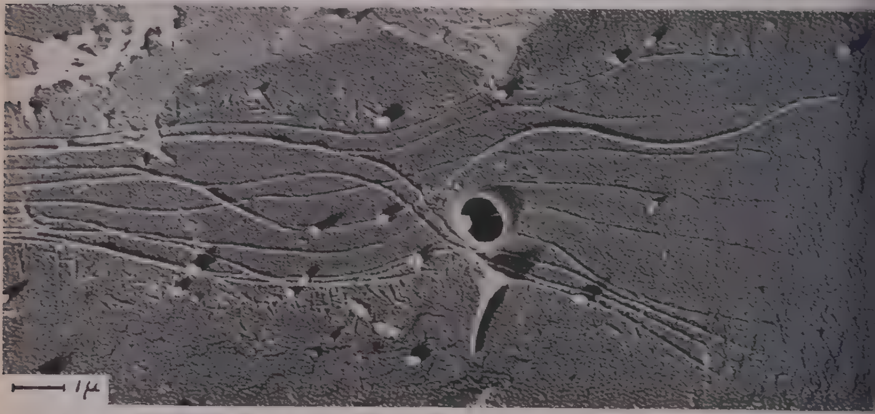
G. 11. *Himanthalia lorea*, tip of a front flagellum showing the spine. Electron micrograph M96·29, magnification × 10,000.

I. 12. More highly magnified part of the preceding showing the bases of the two rows of lateral hairs still in position. Electron micrograph M99·1, 60 kV., magnification × 20,000.

IG. 13. Detail of well preserved *Flimmer* from formalin hardened material. Reversed print of electron micrograph M97·18, magnification × 20,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 5





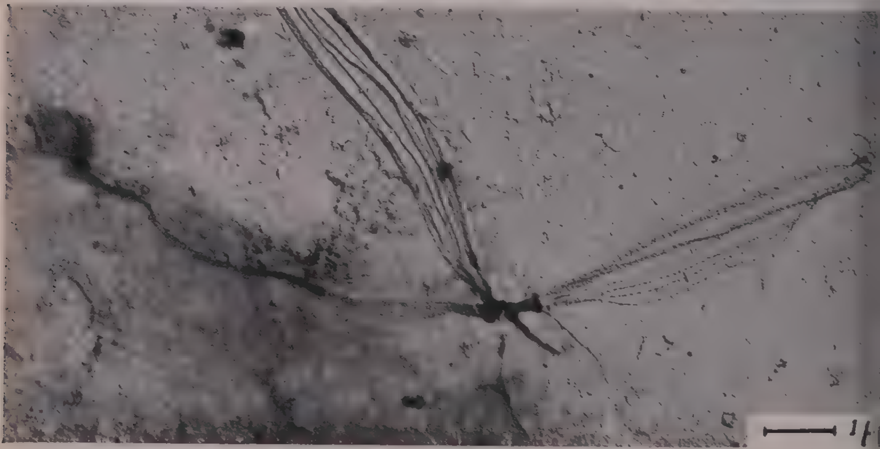
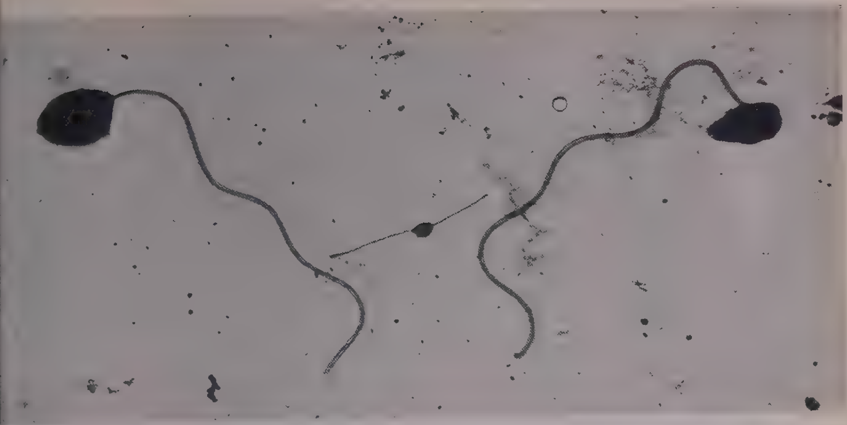


FIG. 14. *Himanthalia lorea*, a spermatozoid with the front flagellum in an early stage of disintegration. The skin is cle visible outside the fibrils. Electron micrograph M92·5, magnification ×8,000.

FIG. 15. Distal end of a completely dismembered front flagellum of the same showing the eleven strands and the p of attachment of the spine. Reversed print of electron micrograph M92·7, magnification ×7,500.

FIG. 16. Part of a completely dismembered cell of the same showing the attachment of the two flagella to each other to the body. Electron micrograph M99·10, magnification ×10,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 6



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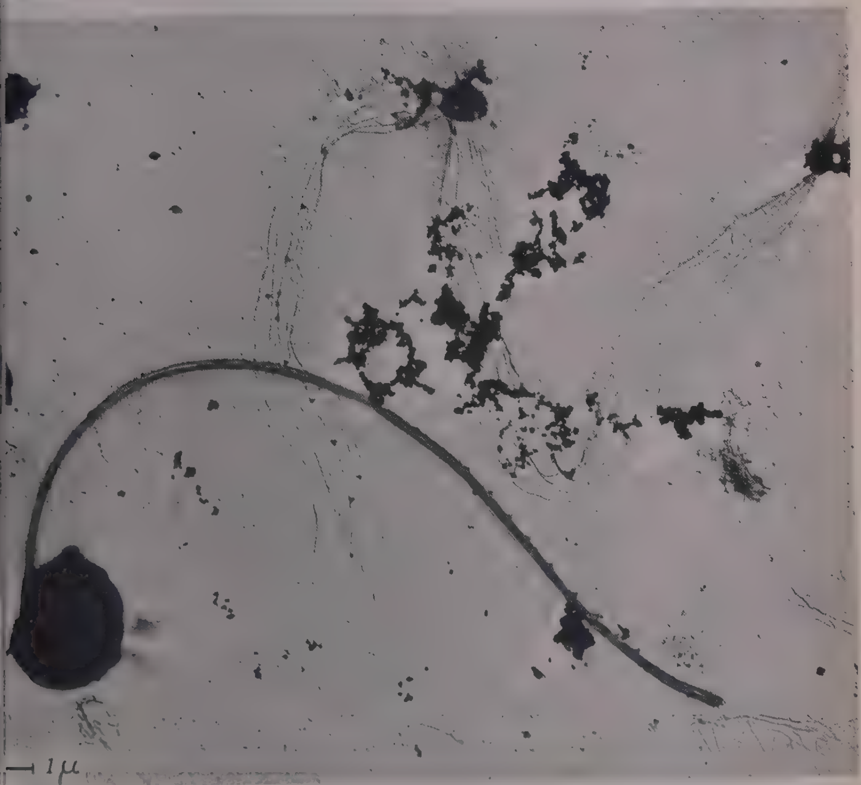

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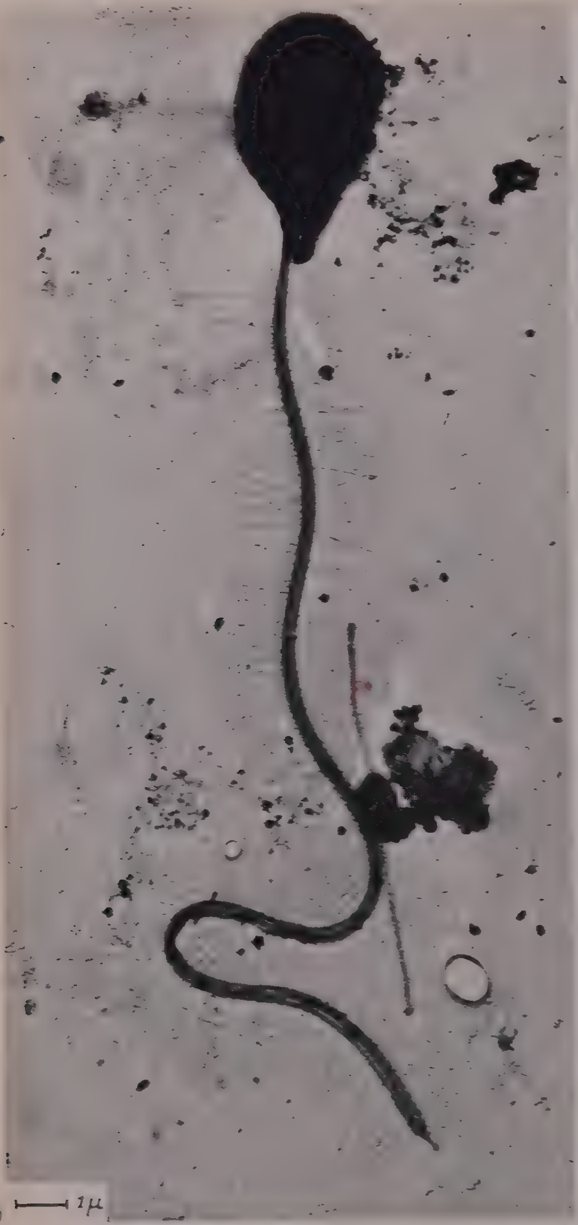
FIG. 17. *Dictyota dichotoma*, two spermatozoids. Electron micrograph M93·30, magnification × 3,000.
18. An intact and a dismembered spermatozoid of the same. Electron micrograph M93·27, magnification × 5,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 7



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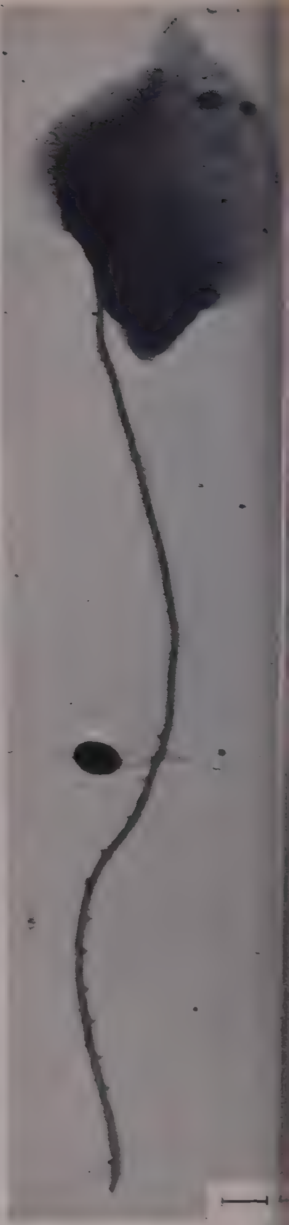
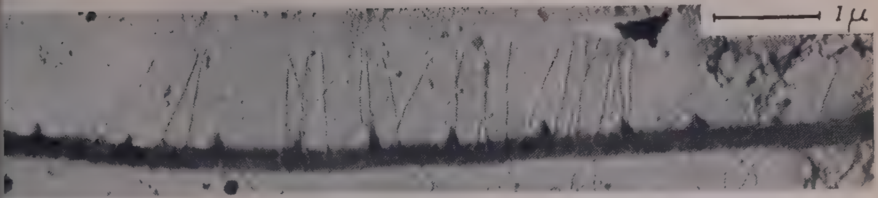


FIG. 19. *Dictyota dichotoma*, a spermatozoid showing the two rows of lateral hairs extending to the tip of the flag
the spines being superposed on the flagellum axis and therefore not visible in silhouette. Electron micrograph M
magnification × 17,500.

FIG. 20. A similar specimen from which the hairs have been shed and in which the body has partially flattened on th
The row of spines is visible in silhouette at the distal end of the flagellum. Electron micrograph M, magnifi
× 8,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 8



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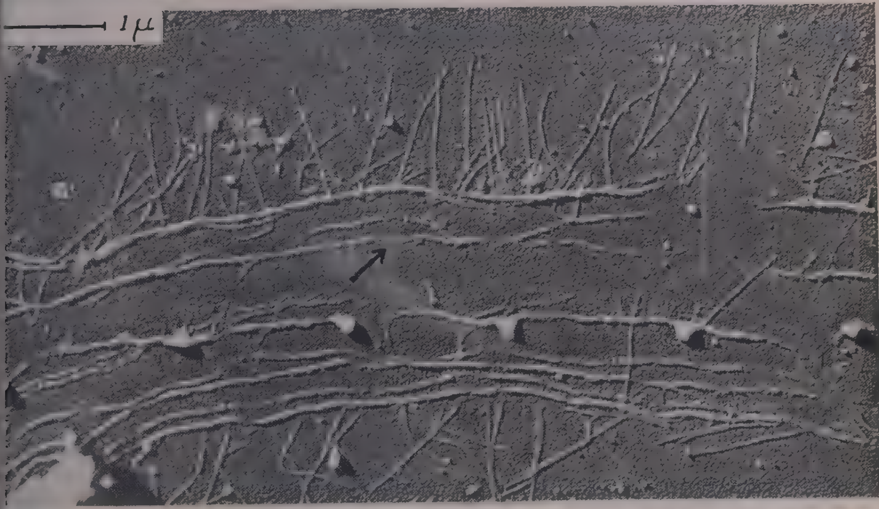

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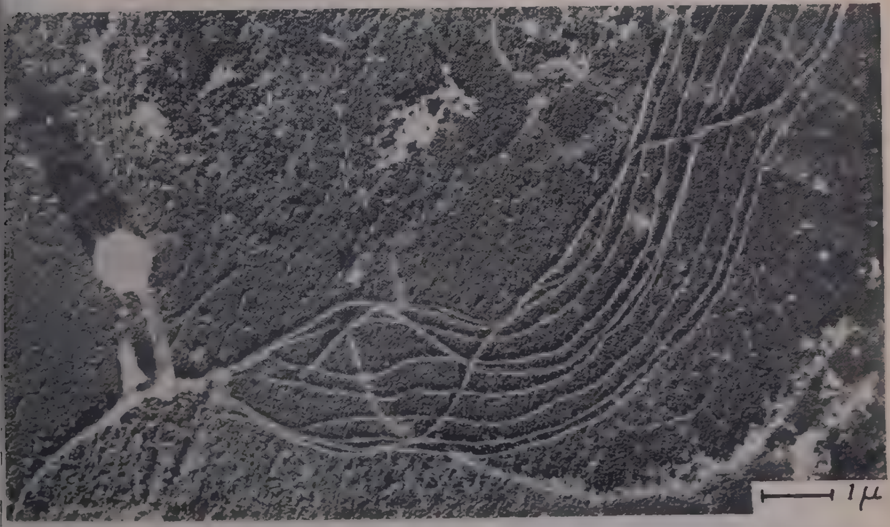

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FIG. 21. *Dictyota dichotoma*, more enlarged view of the tip of a flagellum showing the spines in silhouette and some of the hairs still attached. Reversed print of electron micrograph M99·20, 60 kV., magnification × 15,000.

FIG. 22. Part of a dismembered flagellum in the spine-bearing region. Reversed print of electron micrograph M94·15, magnification × 15,000.

FIG. 23. Base of a dismembered flagellum showing eleven strands and the various appendages inside the cell body. Reversed print of electron micrograph M93·23, magnification × 10,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE 9

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Studies on the Growth and Respiration of Roots

I. THE EFFECTS OF STIMULATORY AND INHIBITORY CONCENTRATIONS OF β-INDOLYLACETIC ACID ON ROOT SECTIONS OF *PISUM SATIVUM*

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SUMMARY

Simultaneous observations on extension growth and respiration rate (oxygen consumption) of 2-mm. sections excised from the extension zone of roots of pea (*Pisum sativum*) growing in distilled water and 0·5 per cent. sucrose have yielded results closely similar to those of Brown and Sutcliffe (1950). Respiration rate is not obviously correlated with growth rate either in water or in sucrose, but it is strongly correlated with section length. Respiration rate per unit section length (= per unit fresh weight) shows a marked downward drift during extension and is affected little by growth conditions. Tentative suggestions are advanced to account for the small differences between drifts in 0·5 per cent. sucrose and those in distilled water.

Medium agitation produces an immediate and sustained stimulation of growth but no stimulation of oxygen uptake until the later growth stages. Thus respiration per unit section length is unaffected by agitation at any stage.

A typical growth response to β-indolylacetic acid (IAA) was obtained, with a maximum stimulation (of about 35 per cent.) at 1 part in 10^{11} and inhibitions increasing progressively with concentration beyond the threshold of about 1 part in 10^{9}. Both percentage stimulation and percentage inhibition of growth were independent of the presence of sucrose.

Respiratory responses to β-indolylacetic acid were complex. In water no immediate response could be detected with either a growth-stimulatory (10^{-11}) or a growth-inhibitory (10^{-8}) concentration, while in 0·5 per cent. sucrose the inhibitory concentration prevented the small immediate respiratory rise due to the sucrose, probably by impeding sugar entry. During the subsequent period of rapid growth (up to 36 hours) the small respiratory responses observed closely followed the small growth responses to both concentrations of IAA, suggesting that the former are the direct result of the differences in section length induced by the auxin. When growth ceases (at 48 hours) sections which have grown considerably in sucrose show respiratory rates still closely correlated with section length, whereas in water both concentrations of auxin induce marked depressions in respiration rate.

It is concluded that β-indolylacetic acid in both growth-stimulatory and growth-inhibitory concentrations has no *direct* effect on the activity of the respiratory enzyme system of growing root cells. The small respiratory responses are best explained as resulting from differential changes in section size and correlated changes in the enzyme complements of the growing cell.

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INTRODUCTION

ER since the pioneer work of Bonner (1933) physiologists have devoted uch attention to the interrelationships of extension growth and respiration d the associated supply of growth energy. For the most part the several vestigators have claimed that auxin applied in physiological concentrations ings about an immediate augmentation of the oxygen uptake of the order 15–30 per cent. although some responses have been closely dependent on e operation of other factors, e.g. prior soaking of *Avena* coleoptile sections malate (see Thimann, 1949). Furthermore, observations that certain hibitors of specific respiratory processes (e.g. iodoacetic acid, arsenic salts, .) will prevent this respiration response have led to the association of auxin tion with particular metabolites or enzyme systems; cf. Thimann, 1949; nner and Wildman, 1947.

Two serious criticisms can be raised against much of this previous work. he first is that the published papers give little indication of the statistical gnificance of the small stimulations of respiration observed. In this labora-ry, experience with stem and root sections of pea has shown that the efficient of variation ($100\sigma/\bar{X}$) of the initial respiration of exactly comparable mples is about 10 per cent. It can easily be shown that this is also the order the least significant difference (5 per cent. point) to be expected between eated and control means of a ten-fold replicated experiment. This is the size stimulation observed in many experiments, mostly having fewer replica-ons than this, and indicates that much of the early work requires checking n this point. The second criticism is that little attempt has been made to bserve auxin effects on respiration and growth simultaneously over the hole growth period. Respiration effects have usually been determined as an verage over the first few hours after excision of the sections, while effects on rowth have been recorded over a much longer period. The dangers of this uperficial approach have already been pointed out by one of us (Audus, 1952).

The particular difficulty here is that any respiratory effect of auxin treat-ent may be either direct on the respiratory system or indirect and arising rom growth changes. By taking average values any respiration drifts are bscured and the only clues for distinguishing between these two possibilities re therefore thrown away. This point can well be illustrated by recent observa-ions on excised root sections in which respiration rises steeply as the cell xtends rapidly in the early growth phases in sucrose solutions. No such rise s seen in slowly extending sections in water (Brown and Sutcliffe, 1950). f respiration rates had been determined as averages for the first few hours the apidly growing material would have shown a considerably higher mean espiration than the slowly growing sections, and this might have been inter-reted as a direct effect of the sucrose on the respiration system. Applying the ame argument, many of the respiration differences recorded as due to the irect action of IAA on coleoptile respiratory systems might be similarly xplained as an indirect result of the increased growth of the auxin-treated sections. In the absence of data on respiratory drifts during growth this poin cannot be settled.

For a variety of reasons it was decided to investigate roots, as the work o Burström and of Brown had given evidence of their suitability and sinc much valuable information on the changes of respiration during growth i excised root sections already exists from the extensive researches of Brow and Sutcliffe (1950), providing a basis on which to plan experiments on th action of auxins. The excised section technique of Brown and Sutcliffe ha been used throughout with a few minor modifications.

The two distinct growth responses of roots to auxins, acceleration belo a threshold of about 1 in 10^9 and retardation at higher concentrations tha this, provide an opportunity to investigate, at levels both stimulatory an inhibitory to growth, the possible associated effects on respiration.

MATERIAL AND METHODS

1. *Materials.* Seeds of *Pisum sativum* (var. 'Meteor', Messrs. Sutton an Sons, Reading) were surface sterilized by 2 minutes' immersion in 80 per cen alcohol followed by 2 minutes in 0·2 per cent. mercuric chloride. They wer then washed in very dilute sodium sulphide solution to precipitate an remaining mercury ions and then washed overnight in aerated running tap water. Germination took place in sterile sand moistened with sterile glass distilled water at 18° C. in a dark cupboard.

Seedlings with straight roots 3·5 to 4·5 cm. long were selected on the thir day after planting when they had single primary roots and no shoots.

2. *Preparation of sections from the extending zone.* Preliminary experiment were carried out to determine (*a*) the optimum length of section, and (*b* the position behind the tip from which to cut it in order to get the maximu subsequent extension growth. These led to the adoption for all the mai experiments of 2-mm. sections cut at a distance of 2 mm. from the extrem tip of the root. For this a double-bladed cutter, producing 12 to 15 section at a time, was employed. It was of essentially the same design as that used b Brown and Sutcliffe (1950).

3. *Culture media.* Preliminary observations were also made on growth i glass-distilled water and in solutions of 0·5, 1, and 2 per cent. sucrose. Eve the lowest of these concentrations gave maximum growth and was therefor chosen for the main experiments since it was felt that at this level the possibility of disturbances due to bacterial contamination of the cultures wa minimized. The use of all buffering salts was strictly avoided since there ample evidence that inorganic ions have profound effects on growth an respiration of roots and root sections (Brown and Sutcliffe, 1950) and ma also greatly modify the responses of tissues to auxins (Thimann and Schneide 1938). The basic growth media used were therefore glass-distilled water an 0·5 per cent. sucrose in glass-distilled water. The pH remained between 6· and 7·0 during the whole of the growth period of 48 hours and also during th respiration determinations in the Warburg apparatus.

4. *Experimental procedure.* A sampling technique was used. After selection o seedlings the roots were removed as a whole and washed thoroughly in g ss-distilled water. The experimental sections were then excised and replaced i fresh glass-distilled water. Random samples of 25 were then transferred to Ptri dishes containing 10 ml. of the culture medium. Dishes of any particular cture medium were replicated sufficiently to allow of subsequent sampling (ually 4 times). The Petri dishes were covered with lids and then placed in th dark in an incubator at 25° C. and rocked gently on a motor-driven tilting dvice. At suitable time intervals selected dishes were removed from the icubator and the samples of sections contained therein transferred each to a Warburg flask together with 1 ml. of the culture medium in which they had ben growing. Subsequent successive 15-minute readings of oxygen uptake oly were taken over periods of 3–4 hours. The Warburg apparatus was in one crner of the laboratory remote from the window and light intensity there was lw, but no attempt was made to exclude all light. Some respiration determinatons were made with some flasks illuminated by two 150-W. tungsten fament bulbs immersed in the bath while others were covered by black vlvet bags. They demonstrated that neither growth, respiration, nor auxin rsponses were affected by this range of light intensity.

After their respiration rates had been measured, the sections were removed fom the flasks and their lengths measured to the nearest 0·01 mm. under a tavelling microscope. No appreciable changes in diameter occurred under te conditions of the experiments and so length increases were taken as the sle measure of growth. The sections were then discarded.

This order of procedure was regarded as advisable since preliminary oservations indicated that the relatively vigorous agitation in the Warburg fsk under conditions slightly different from those of the incubator had little efect on section elongation, whereas exposure of the sections during length easurements to the cold conditions of the laboratory bench (18° C.) did hve an effect on subsequent respiration.

The culture medium remaining behind in the Warburg flask after the rmoval of the sections was subjected to routine measurements of the pH and rsidual oxygen uptake, the latter being an indication of the degree of contmination by micro-organisms. Fortunately contamination was never found any sample before 36 hours and in a very few after this time. Results from ch contaminated samples were discarded.

5. *Growth conditions.* There is ample evidence to show that roots are articularly sensitive to oxygen in the medium and their growth can be very arkedly reduced by even slight deficiencies of it (Brown and Sutcliffe, 1950). oult (1939) demonstrated that agitation could markedly stimulate the growth f *Sinapis alba* roots and attributed this to increased aeration.

Preliminary factorial experiments were therefore carried out to determine he full effect of aeration by agitation on section growth and respiration. 5 and 2 per cent. sucrose and water controls were studied. A growth-imulatory concentration of IAA (1 part in 10^{11}), an inhibitory one (1 part in 10^8), and controls without auxin were used and all nine possible com binations of these treatments were investigated. Growth was followed ove 0–12, 12–24, 24–36, and 36–48 hour periods after excision and respiratio determined for the same samples at the ends of these periods. In all treatment one set of samples was studied in an undisturbed (still) medium while a exactly similar set was agitated by rocking at the rate of two oscillations pe minute through an angle of about 10° C. This tilting movement meant th all sections were completely drained of medium and then covered with once every oscillation. Maximum aeration was thereby attained with minimu mechanical disturbance of the sections.

Naturally such a multifactorial investigation could not be embraced by o single 2-day experiment. Since, however, individual experiments were a performed within a short time interval and under strictly comparable co ditions and with the same batch of pea seeds,[1] the data have been lumpe and subjected to an analysis of variance. As a result, between-experime variance has been confounded with residual error, but this should not obscu the main effects of treatment.

The following table (Table I) shows the results of this analysis. Only t main effects and interactions which reach the 5 per cent. level of significan have been tabulated.

TABLE I

Growth rate per section in mm./12 hours

Source of variance	Sum of squares	Degrees of freedom	Mean sq. variance	P
Sugar (S)	5·807	2	2·91	—
Auxin (I)	1·289	2	0·645	—
Rocking (R)	6·096	1	6·096	—
Time (T)	10·469	3	3·49	—
Interactions:				
S×R	2·044	2	1·022	<0·01
S×T	3·932	6	0·655	<0·01
I×T	2·562	6	0·426	<0·05
R×T	2·757	3	0·917	<0·05
Residual	7·836	46	0·170	—

Respiration rate in μl. oxygen/30 min./25 sections

Source of variance	Sum of squares	Degrees of freedom	Mean sq. variance	P
Sugar (S)	516·5	2	258·2	—
Auxin (I)	583·5	2	291·7	—
Rocking (R)	226·3	1	226·3	—
Time (T)	1,431·3	4	357·8	—
Interactions:				
S×R	296·5	2	148·2	0·001
S×T	662·9	8	82·8	<0·001
R×T	531·8	4	133·0	<0·001
S×T×R	379·9	8	47·5	0·02
Residual	1,101·7	58	19·0	—

[1] Difficulty has sometimes been experienced in that apparently similar batches of pea se obtained in the same year from the same seed merchant show appreciably different gro behaviour. *Within* a particular batch, however, no such variability is found.

Since we are concerned here only with the effects of rocking, we need not onsider the characteristics of the effects of auxin and sugar and the time ifts, except in so far as they are affected by rocking. These tables show that ocking has a highly significant effect on both growth and respiration per ction and that its interactions with time and with sucrose concentration are so highly significant. These effects are illustrated in Fig. 1. The growth

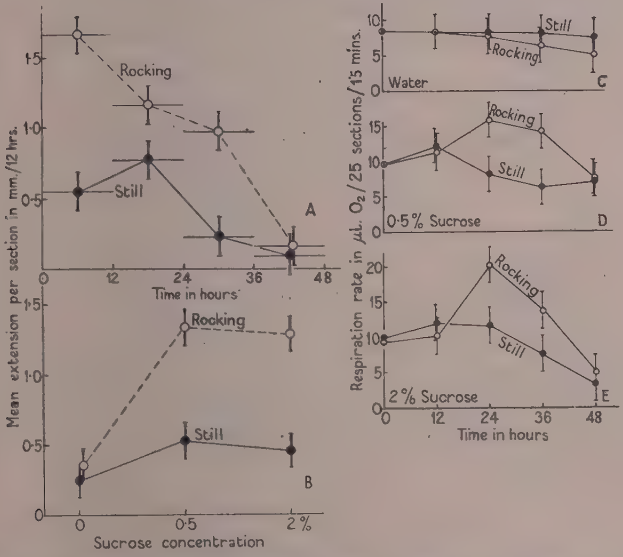

FIG. 1. Graphs showing the effects of medium agitation on growth rate and respiration rate in excised pea root sections growing in water and two concentrations of sucrose. Only significant main effects and interactions have been drawn. The vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of the means, σ having been calculated from the residual mean square variances of Table I. (For further explanation see text.)

ate is greatly stimulated by rocking during the whole of the extension of the ections (Fig. 1, A), the most marked effect (a threefold increase) being pro-uced in the first 12 hours. Turning to its relationship to sucrose concentration Fig. 1, B) there is a slight growth stimulation in water, but this does not reach he required level of significance. In both concentrations of sucrose, which will e seen to stimulate growth to the same extent, the effect is much greater and ighly significant. This suggests that a most important aspect of the rocking ffect may be its facilitation of the utilization of sucrose in growth. In contrast o its marked effect on growth in the first 12 hours, rocking has no significant ffect on respiration over the same period (Fig. 1, C–E) in any sucrose con-entration. Clearly then the effect is not due to the action of increased aeration n the oxidation of sugars and the resultant provision of growth energy. n water the rocking effect never reaches an acceptable level of significance.

In both sugar concentrations, however, the main effect is marked by a doubling of the respiration per section at 24 to 36 hours. As we shall see when th main data are considered, these peaks can be best explained in terms of th increased size of section produced by the rocking, since an alternative analysi of variance of this data shows that the respiration *per unit length* of section i.e. a direct measure of the respiration per unit fresh weight, is indistinguishable for sections in agitated and in still media at these times and that th holds whether the sections are in water or in sucrose. The relevant means the respiration per unit length are recorded in Table II below.

TABLE II

Mean oxygen absorption in μl./hour/mm. length

	Still medium	Shaken medium	Difference
24 hours	0·497	0·489	0·008
36 hours	0·361	0·338	0·023

Least significant difference at the 5 per cent. level = 0·095

The full significance of these results will be considered later. One interes ing feature of these analyses is that the auxin effects are completely indepe dent of the rocking. Because of the markedly improved growth brought abo by the agitation, in all the main experiments this type of rocking was adopte as a routine procedure.

EXPERIMENTAL RESULTS

Growth and respiration drifts in water and in 0·5 per cent. sucrose in the absen of β-indolylacetic acid

Many control observations on the growth and respiration of sections both glass-distilled water and in solutions of 0·5 per cent. sucrose have be made during the course of the auxin investigations. It is a characteristic the sampling technique used that these results are subject to considerab inter-sample variation, necessitating the performance of many experiments establish small treatment differences. The coefficients of variation of bo growth rate and respiration rate per section between individual experimen were of the order of 10–15 per cent. For sets of samples taken from the sar batch of pea roots grown on the same day it was considerably smaller, seldo rising above 10 per cent., and use is made of this when analysing the ma auxin effects. The results of Brown and Sutcliffe (1950) suggest that the material was more uniform, the coefficient of variation for growth and respir tion being about 5 per cent. The reason for this difference is rather obscu There may be a higher intrinsic variability in pea roots of the particular varie we used. Another suspected source of this increased variability was the co ditions of germination of the seedlings; the temperature was not rigorous ntrolled but varied between 18° C. and 20° C.[1] The data so collected have en presented in Figs. 2 and 3.

Fig. 2 shows the extension results, each point representing the percentage tension of one sample after the relevant time interval. The two curves for

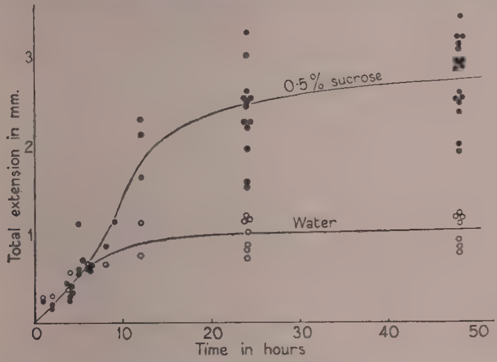

FIG. 2. Extension growth of 2-mm. sections of pea roots growing in distilled water and in 0·5 % sucrose. The curves have been fitted freehand.

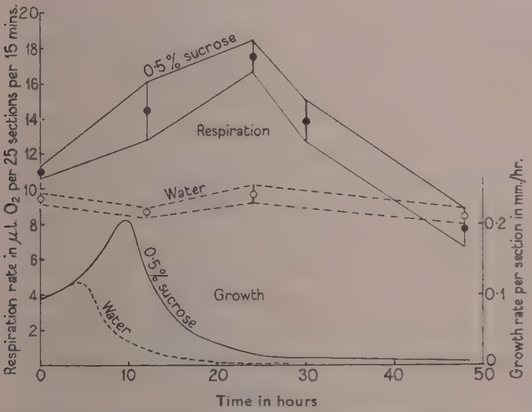

FIG. 3. Graphs showing the changes in respiration rate per sample of 25 root sections during extension growth in both distilled water and 0·5 % sucrose. In the respiration results the vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of the relevant means. The growth rate curves have been measured directly from the slopes of the curves of Fig. 2.

growth in water and 0·5 per cent. sucrose have been drawn freehand. A umber of interesting points emerge from these curves. Firstly growth rates n both water and sucrose follow the same time course for the first 6 hours, suggesting that the sections contain sufficient carbohydrate reserve to allow maximum growth rate over this period. It is unlikely, from Brown and Sutcliffe's observations on the time course of sugar absorption in such sections, that this can be entirely explained by the failure of appreciable quantities of sugar to enter the sections in this time. This view is supported by the respiration results discussed below. From this time on, the growth rate in sucrose continues to increase to a maximum at about 10 hours while that in water falls away rapidly. Indeed in water growth virtually ceases at about 20 hours In sucrose, on the other hand, appreciable growth is still going on at abou 48 hours after excision. Data obtained with another technique and no presented here shows that growth usually continues until 60 or 70 hour have elapsed after cutting. This stimulating effect of external sugar on th growth rate and its prolongation of the total period of growth are mos probably due to the additional sugar made available in the tissues, although th stoppage of growth in water is not due to the complete exhaustion of sugar in the cell, since Brown and Sutcliffe have demonstrated that in such section the internal sugar concentration falls to only a third of its initial value afte 48 hours. It is possible that the extra sugar, in addition to promoting increase synthesis of cell materials in growth, brings about some change in th metabolic balance in the cell such that either an inhibitor causing earl stoppage of growth or a deficiency of a necessary growth factor is removed

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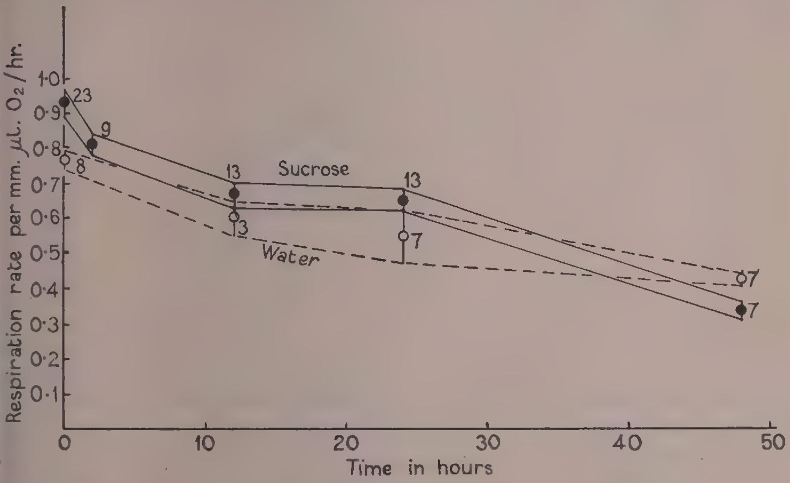
[1] More recent experiments, where germination temperatures have been rigorously con-rolled at 25° C. and humidity held at near saturation, have reduced the coefficient of variation o about 10 per cent., i.e. to the within-sample figure. There seems little hope as yet that it nay be reduced below this value for the particular material employed.

The respiration data appear in Fig. 3. In water the respiration, expresse on a per section basis, shows slight but significant ($P = 0{\cdot}1$) changes ove the 48 hours. Individual '*t*' tests on pairs of means show that the sligh maximum at 24 hours is the most significant fluctuation ($P = 0{\cdot}05$). In 0· per cent. sucrose the respiration immediately after cutting is significantl higher than in water (16%). This shows that sugar in some form must ent the cell in appreciable quantities immediately after immersion in the solutio since the effect is observed in the first hour. The oxygen absorption per sectio increases steadily for the first 24 hours to a maximum about 60 per cen higher than the initial level. Thence onwards it declines rapidly and at hours, when growth has become very slow, it has been reduced to the lev of the respiration of the much smaller sections in distilled water. The mo interesting feature of these results is that this respiratory maximum occu when the growth rate has declined to roughly 12 per cent. of its maximu value, which occurred 14 hours earlier at 10 hours from excision. The results resemble those of Brown and Sutcliffe who found a similar peak respiration at 12 hours in 0·75 per cent. and at 36 hours in 2 per cent. sucros the growth maxima occurring at both these concentrations in the first 4 hou after section excision.

Clearly sucrose has a marked effect on the respiration of these growi sections, the degree of stimulation being very closely dependent on the sta of growth. There is, however, no close correlation with the growth *rates* (s Fig. 3). Nevertheless it is difficult to see what precise meaning we can gi to these 'per section' respiration results when it is remembered that we a ealing here with a rapidly changing metabolic system, even in the sections rowing in distilled water. Much data now exists to show that protein nthesis takes place in the extension zone of intact roots during this phase cell growth. Brown and Broadbent (1950) have shown, also in pea roots, at the total protein nitrogen per cell increases to a maximum as cell volume creases and then declines as the cell matures after extension. The rate of



IG. 4. Graphs showing the change in respiration rate per unit length (≡ unit F.W.) of xtending root section during extension growth in both distilled water and 0·5% sucrose. he vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of the relevant means. The relevant number of samples studied is recorded by each mean.

xygen uptake per cell runs closely parallel. It is possible that the two are ausally related, the quantity of respiratory enzymes being a fairly constant roportion of the total protoplasmic proteins. These enzymes may probably e saturated with substrate under normal conditions. The situation in isolated ections extending in distilled water or sucrose is somewhat different since here is no external source of nitrogen for synthesis of additional protein. Nevertheless it seems highly probable that the general pattern of protoplasmic structural proteins must change considerably during extension growth n such sections, as shown by the dramatic changes in enzyme activity, particularly invertase, during maturation of the cells of the root-tip (Brown, Reith, and Robinson (1952). One aspect of this change in protoplasmic pattern could easily be an augmentation of the respiratory enzyme system and there is no reason why this should not take place also in isolated extending segments. If this were so it would give a most rational explanation of the rise in respiration rate during the early stages of rapid growth in these sections.

The only way with the present data of offsetting changes in respiration due o the possible growth of protoplasm during extension is to recalculate the respiration on a fresh weight basis, i.e. on a unit section length basis, which is its equivalent in the present material, since section length is a direct measure of fresh weight. These recalculated respiration rates are illustrated in Fig. 4.

The obvious feature of these new curves is that the respiration per unit length in sucrose is little different from what it is in water. Both curves decline steadily from the time of excision to the end of the experimental period (48 hours). '*t*' tests have been carried out where possible on pairs of means to determine the significance of these sucrose-water differences. The results are given in Table III.

TABLE III

Respiration rate per unit length of section. Differences between sucrose and water values (μ*l.* O_2*/mm./hr.*)

Time (hours)	Difference (μl. O_2)	Ratio: Diff. Water mean	'*t*'	*P*
0	0·156	0·205	2·45	0·02
12	0·070	0·12	0·89	—
24	0·102	0·19	2·25	0·035
48	−0·089	−0·21	2·91	0·1–0·2

Thus during the period of rapid growth in sucrose the respiration per uni fresh weight in sucrose remains slightly above that in water. At 0 and 24 hour this difference is clearly significant, but it falls somewhat below the significance level at 12 hours. After 24 hours, however, when most of the growth i over, the respiration in sucrose falls steeply to a level as far below that of th water control as it had previously been above it, and this difference is als significant.

A tentative explanation of these somewhat complex results is that durin extension growth in any medium the respiratory enzyme system of the cel is constantly being augmented and is some simple function of cell size. Th material for this enzyme synthesis could come from non-protein reserves o by breakdown of other structural cell proteins. The essential point is that th general pattern of protein structure in the cell is constantly changing durin extension (see Brown *et al.*, 1952). At the same time and as the result of ce maturation processes, which are to a large extent independent of cell elonga tion, the respiratory activity per unit respiratory enzyme declines slowl during extension. This maturation 'activity suppression' is based on the sam concepts as Brown's 'arresting mechanism', which was evoked to explain th cessation of extension growth itself at the end of the growth cycle in the cel It seems unlikely that this downward drift is the result of substrate concentra tion changes, since it is not arrested at all by the external provision of sucros which probably causes a steady increase in the internal sugar concentratio (Brown and Sutcliffe, 1950).

The effects of β-indolylacetic acid on the growth and respiration drifts

The effects on root growth of β-indolylacetic acid applied in the extern medium are now well established by many independent observations. Ro sections of pea show a considerable stimulation of growth in very lo concentrations (1 part in 10^{11} and 10^{10}) and a progressive inhibition in increasng concentrations above 1 part in 10^9. Results of typical experiments on 5 amples of 25 sections, each in a different concentration of the acid, are llustrated in Fig. 5. The stimulation here is average and of the order of 20–25 per cent.

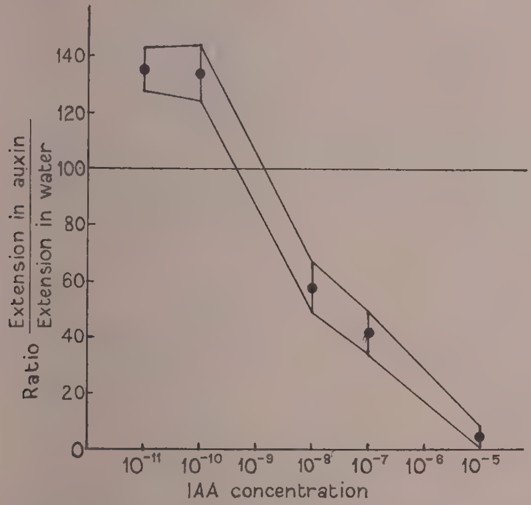


FIG. 5. Growth response of excised pea root sections to a range of β-indolylacetic acid (IAA) concentrations. Each point represents the grand mean from three samples, each of 25 sections. The vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of these means, σ having been derived from the variance of individual sample means about the grand mean.

It was decided in the main investigations to employ only those concentrations of auxin which might be justifiably termed 'physiological', i.e. those giving stimulations and inhibitions of extension rate normally associated with the action of natural auxins in the growth of roots. All criticism that toxic effects were being obtained in high concentrations would then be avoided. Two concentrations were therefore chosen; one, corresponding to the usual peak stimulation value, was 1 part in 10^{11}. Concentrations as low as 1 part in 10^9 often give inhibitions, but the variations in response at this concentration level, where the curve crosses the no-response line, are very much greater than at other concentrations and inhibitions are not consistently obtained. One part in 10^8 with its consistent inhibitions was therefore selected as the second experimental concentration.

Under all experimental conditions, whether studying the effects of one or both concentrations of auxin in the presence or absence of sucrose, control samples without auxin were always observed at the same time and all subsequent responses of growth or respiration calculated and expressed as ratios to the corresponding controls. In this way the total variability of the data has been reduced. Although the within-batch variance remains, the between-batch variance is eliminated in so far as the treatment effects themselves are independent of batch. The coefficient of variation here is about 10 per cent.

(*a*) *Growth results*. Data have been accumulated for the total extension of 25-section samples at 12, 24, and 48 hours after excision. Roughly equa numbers of experiments have been carried out in distilled water and in 0·5 per cent. sucrose solutions and on each occasion the effects of both concentrations of β-indolylacetic acid were followed. The mass of data as i stands is not suitable for immediate analysis, but by random omission of a few experimental sets it has been made symmetrical with respect to the thre mean variables (time, sucrose, and auxin concentrations) and has been subjected to an analysis of variance. The results of this procedure are brough together in Table IV.

TABLE IV

Ratio of extension in auxin extension without auxin.

Source of variance	Sum of squares	Degrees of freedom	Mean sq. variance	*P*
Auxin conc. (I) . .	3·8905	1	3·8905	<0·001
Sucrose conc. (S) . .	0·0711	1	0·0711	—
Time (T) . . .	0·3867	2	0·1933	0·15
Interactions:				
I×S . . .	0·0114	1	0·0114	—
I×T . . .	0·1246	2	0·0623	—
S×T . . .	0·1135	2	0·0567	—
Residual . . .	5·7443	55	0·1044	—

The auxin effect is the only one which is highly significant. Sucrose completely without effect and its interaction with auxin concentration also insignificant. This means that auxin stimulations and inhibitions (se Fig. 6) are exerted to the same extent whether sections are growing slow in distilled water or rapidly in 0·5 per cent. sucrose solution. This probab means that both auxin inhibitions and stimulations of extension growth a not connected in any way with the utilization of the carbohydrate substra in the growth of these root sections. There is a slight effect of time whic approaches the significance level, and this time effect is independent of eith auxin or sucrose concentration. The nature of this effect and the ma β-indolylacetic acid effects are illustrated in Fig. 6. The consistent growt rate stimulation of about 30 per cent. in 10^{-11} auxin and the inhibition about 20 per cent. by 10^{-}[illegible] auxin are clearly demonstrated. The small tir effect consists of a small reduction in response in the stimulatory concentr tion and a small increase in inhibition in the higher concentration. In oth experiments using a slightly different technique (Audus and Shipton, 195 a much more rapid decline in response was observed in the stimulato oncentrations. This fall may to some extent be due to a loss of β-indolylcetic acid from the medium (by bacterial action) and from the tissue of the oot by enzymic inactivation. Indirect 'maturation' effects may also be playing part in these time drifts of apparent response to auxin treatment.

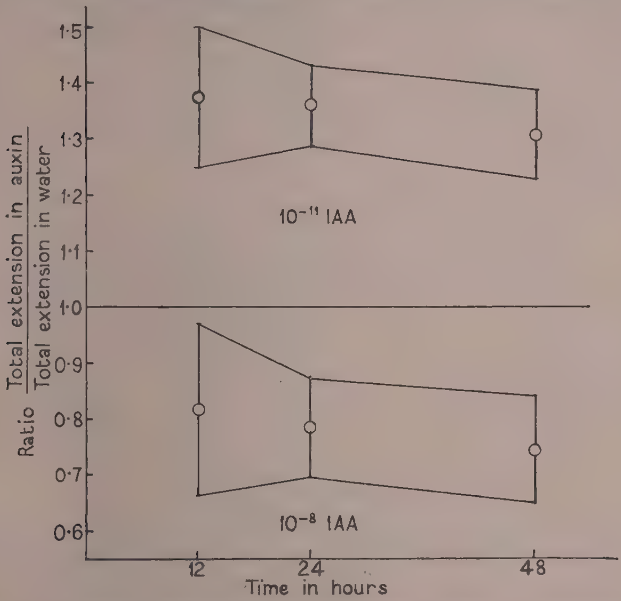


FIG. 6. Analysis of growth responses of pea root sections to stimulatory (1 part in 10^{11}) and inhibitory (1 part in 10^{8}) concentrations of β-indolylacetic acid (IAA). The vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of the means, σ having been calculated from the residual mean square variance of Table IV.

(*b*) *Respiration results*. Precisely the same methods have been employed for the analysis of the respiration results. The data used were the measurements of the respiration rate per section. The results of the analysis of variance are given in Table V.

TABLE V

Ratio: Respiration rate in auxin/respiration rate without auxin

Source of variance	Sum of squares	Degrees of freedom	Mean square	*P*
Auxin concs. (I)	0·4796	1	0·4796	0·01
Sugar concs. (S)	0·0544	1	0·0544	—
Time (T)	0·3918	3	0·1306	0·03
Interactions:				
I×S	0·0361	1	0·0361	—
I×T	0·2285	3	0·0762	0·2
S×T	0·5678	3	0·1826	0·01
Residual	2·8610	72	0·0397	—

Clearly again there is a highly significant effect of β-indolylacetic acid concentration, and the time effect, in contrast to that on growth, is quite significant. The large time interactions with both sugar and auxin concentrations show that further analysis is necessary. The nature of these interactions and the main effects are illustrated in Fig. 7. These curves, being ratios of the respiration rates in auxin to those in water, represent the modifications in the drift curves of respiration per section, as they appear in Fig. 3, brought about by the two auxin concentrations.

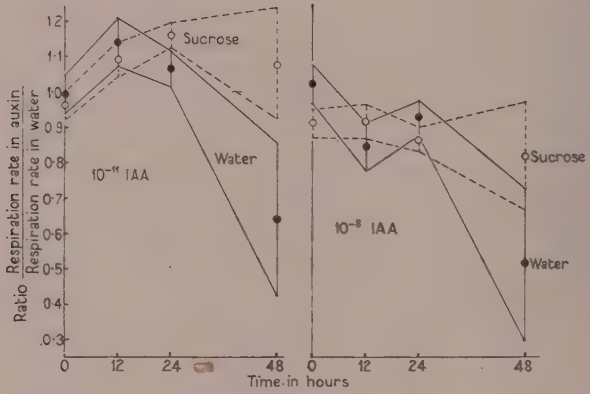


FIG. 7. Analysis of respiration responses of pea root sections to stimulatory (1 part in 10^{11}) and inhibitory (1 part in 10^{8}) concentrations of β-indolylacetic acid (IAA). The vertical lines extend to a distance of $\sigma/\sqrt{N}$ on either side of the means, σ having been calculated from the residual mean square variance of Table V.

Taking first the initial respiration rates immediately after excision of the sections, it will be seen that the growth-stimulating concentration of auxin (10^{-11}) has no effect on the respiration rate per section. The concentration inhibitory to plant growth (10^{-8}), although it has no effect on respiration in distilled water, reduced that in 0·5 per cent. sucrose by about 8 per cent. This reduction is statistically significant at the 1 per cent. point ('*t*' test). It is of the same order as the stimulation produced by sucrose (see Fig. 3) and can be most simply explained by an inhibition, by this concentration of auxin of the entry of sucrose into the sections thus preventing the associated increase in respiration.

Subsequently during the phase of most rapid elongation, i.e. 12 to 2 hours, the effects of auxin on the respiration rate is closely correlated with its effects on growth extension (see Fig. 6) both in water and in sucrose. In 10^{-1} β-indolylacetic acid there is an increase of the order of 10 per cent., while in the concentration of 10^{-8} there is a decrease of about the same size. This suggests that these respiration effects are the direct result of the differences in size of the sections brought about by the augmented or inhibited growth in the relevant auxin concentration. In order to test this more closely the orrelations existing between the individual respiration ratios and their ssociated section length ratios have been worked out. A scatter diagram of he results and fitted regression lines are drawn in Fig. 8. It will be seen that he points clearly fall into two groups, those for the sections grown in disilled water and those for the sections in sucrose. Both these sets of points are ery closely correlated, the water ratios giving a correlation coefficient of $r =$ $\cdot 79$ ($P = 0{\cdot}002\%$) and the sucrose ratios an $r = 0{\cdot}90$ ($P = 0{\cdot}001\%$). The

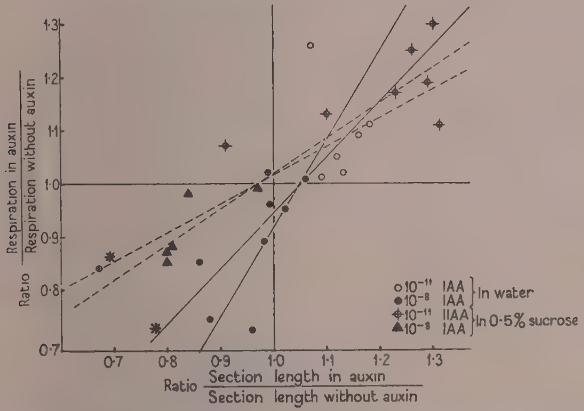


FIG. 8. Correlations between growth and respiratory responses to β-indolylacetic acid (IAA) in excised root sections of pea. (For further explanation see text.)

equations for the two sets of regression lines are: Water ratios: $R = 0{\cdot}077+1{\cdot}026L$. $L = 0{\cdot}446+0{\cdot}602R$. Sucrose ratios: $R = 0{\cdot}469+0{\cdot}551L$. $L = -0{\cdot}513+1{\cdot}480R$, where R is the respiration ratio and L is the length ratio. Both the sucrose regressions differ significantly ($P = 0{\cdot}01$) from the corresponding water regressions, but the differences due to intersect are within the limits of error. The relationship between the effects of auxin on respiration and those on total length is thus different in sucrose from what it is in water. Tests on individual regression coefficients reveal that for the sucrose results they both differ significantly from 1. For the water results one coefficient (the regression of L on R) is just significantly different from 1 at the 5 per cent. point while the other (the regression of R on L) is not.

Inspection of Fig. 8 reveals that these differences are due very largely to the results from the inhibiting concentrations of auxin. We are most concerned, however, with the changes in R produced by changes in L (i.e. regression of R upon L). These regressions are starred in Fig. 8. In water this does not differ significantly from 1 and we can therefore conclude that respiration effects in water can be explained entirely in terms of differences in cell length, i.e. that the respiration per unit length is unaffected by auxin. In sucrose the relevant regression coefficient differs significantly from 1 ($P < 0{\cdot}02$), and we can conclude from this that respiration levels in these inhibited sections are higher than would have been expected from the corresponding extension inhibition data. The extent of this effect is very slight; rough calculations from Figs. 7 and 8 show that it is of the order of 5 per cent. of the net respiration rate. The ultimate reasons for these differences are obscure. Further theorizing along the lines of the above argument would be premature, since it would be based on the assumption that during this phase of the growth cycle the relationship between respiration and cell size is a strictly linear one. This is not so since previous considerations (see Fig. 4) have shown that a slow decline in the respiration per unit length is going on continuously during extension growth.

Returning to Fig. 7 we see that after 24 hours, when the growth rate has fallen to very low values, the respiratory behaviour becomes much more erratic as shown by the great increase in variability of the response ratio. This may be due in some part to two factors: firstly to small increases in oxygen consumption due to bacteria growing on the cut ends of the sections, and secondly to morbid non-uniform changes in the tissues due to the death of damaged cells. Nevertheless from routine inspections and other considerations these effects are not large and it is not thought that they obscure the main trends of the mean values. In sucrose the effects of auxin upon respiration continue to follow closely the effects on section length, i.e. the respiration per unit length does not seem to be affected by auxin even at this late stage. In water, on the other hand, the ratio falls to a very low value a 48 hours in both auxin concentrations. These changes are statistically significant, but the differences due to auxin concentration are not. Thus in water when elongation has stopped, auxin at either concentration induces a much more rapid fall in the respiration drift. This holds for both the respiration per unit length and the respiration per section, since changes in length are negligible over this interval. If, as has been previously suggested, some kind o progressive inactivation of the respiratory enzymes of the ageing cell induce these drifts, then both auxin concentrations would seem to accelerate thi process in these latter stages. We have already seen (Fig. 7) that this deactivation seems to be more rapid in sucrose and this may account for the apparen lack of any additional action of auxin when sucrose is present. This viewpoin is strengthened when we look at the figures for the respiration per unit lengt (Table VI).

TABLE VI

Mean respiration per unit length of section at 48 hours (μl. O_2/mm. hr.)

	β-indolylacetic acid conc.		
	0	10^{-11}	10^{-8}
Water . . .	0·425	0·267	0·283
0·5 % sucrose . .	0·335	0·233	0·264

An analysis of variance of the original data shows that the auxin effect is highl significant at the 0·1 per cent. point but that the sucrose effect is significan only in the absence of auxin. The presence of auxin would seem to brin about the maximum 'inactivation' effect and sucrose cannot increase it furthe

CONCLUSIONS

The first important point which emerges from the experiments described bove is that changes in the oxygen consumption of excised pea root sections owing in both water and sucrose solutions are not correlated with changes *growth rate*. Such correlations would have been expected if the energy equirements of growth itself represented an appreciable proportion of the otal energy made available by respiration. Then changes in this consumption ate might be directly reflected in the respiration drift levels. Instead respiraon rate is correlated with the total section length, or section fresh weight, nce the two run very closely parallel in these extending sections. These espiration per section–section length correlations are superimposed upon a eneral downward drift of respiration, which is regarded as one facet of the ells maturation and an aspect of what Brown has called enzyme inactivation ith age (Brown, Reith, and Robinson, 1952). They are best explained by ostulating that during cell extension there is a new synthesis of respiratory nzymes running closely parallel with the growth in area of the cell wall. hese respiration changes are thus the direct result of growth changes. The espiratory energy potential is probably greatly in excess of actual requirements Frey-Wyssling, 1950; Levitt, 1948), and therefore, during the period of most apid synthesis, active energy diversion into growth has no resultant effect n the total energy production.

The sucrose concentration used has a small effect on respiration *per se*. his is distinguishable as an increase in oxygen consumption per section mmediately after excision and before growth has progressed very far. It is lso maintained as a similar small increase in the level of respiration per unit ength during the period of active extension (i.e. up to 24 hours). Subsequently t is lost, since the downward drift of the respiration per unit length steepens n the sucrose-grown sections and falls well below that of sections grown in vater. This may be due to a more rapid respiratory ‘enzyme inactivation’ in hese sections that have grown and matured more rapidly.

This interpretation of the dual effect of sucrose on respiration, i.e. a basic substrate concentration’ effect and an indirect effect resulting from increased ection size, is strongly supported by the results of the early experiments on nedium agitation. It was shown that whereas rocking greatly augmented rowth and that the major effect was noticed in the first 12 hours, no signiicant effect on the respiration per section could be detected over this period. Significant stimulation of respiration was found only at 24 and 36 hours when he sections had reached the maximum length, and this increase was exactly orrelated with increase in section size brought about by the rocking.

The effects of auxin, the major concern of this investigation, were also xplicable strictly in the same terms. No direct action of auxin, either at growth-stimulatory or growth-inhibitory concentrations, could be detected on the respiration of sections in the first hour or so after excision. The higher growth-inhibiting concentration of 10^{-8} prevented the small respiratory increase in 0·5 per cent. sucrose, probably by interfering with sugar entry. Subsequently small stimulations and inhibitions were observed in the respiration per section relative to controls in the absence of auxin. These responses were also very closely correlated with similar responses in section length an may also be regarded as the direct result of such changes. There is no evidenc whatever that auxins, whether in water or sucrose, have any direct effect o the respiratory enzyme system of root sections, in contrast to the claims mad for effects in *Avena* coleoptiles and pea stem sections. This suggests that muc of the work on these latter structures should be repeated and full attentio paid to correlated section growth. It is quite possible that much of th stimulation of section respiration attributed to a direct effect of auxin on th respiratory system of this material may be due to indirect effects arising fro respiratory enzyme synthesis in the normal course of the augmented growth

There does, however, seem to be an indirect effect of auxin discernible i root sections in the last stages of extension, where the downward drift o respiration per unit length is significantly more rapid in the presence o both concentrations of auxin. This may also have its explanation in a mor rapid 'age inactivation' of respiratory enzymes brought about by the actio of the auxins.

These conclusions are based on experiments carried out under relativel restricted conditions of growth and may be susceptible of criticism on tha score. A study of wider scope, including the employment of other growth an respiration stimulants and inhibitors both with and without auxins, i indicated. Some studies along these lines have already been carried out an will form the subject of subsequent communications.

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The Effects of Certain Growth-regulating Substances on the Rhizomes of *Aegopodium podagraria*

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SUMMARY

1. The effects on elongation and geotropic behaviour of the immersion of rhizomes of *Aegopodium podagraria* for periods of 45 minutes in solutions of β-indolylacetic acid, 2 : 4-dichlorophenoxyacetic acid, ascorbic acid, 2 : 3 : 5-triiodobenzoic acid, and 2 : 4-dichloranisole were investigated.

2. The experimental treatment was carried out in absence of visible light, the rhizomes being photographed every hour by infra-red radiation.

3. After immersion in 10^{-4} M. IAA, the rate of growth is approximately doubled. About 5 hours later it declines to one-third or less of its original value and then gradually recovers. The depression in the growth rate can be reversed by a second immersion in IAA, indicating that it is not due to exhaustion of some other substance essential for growth.

4. Immersion in 10^{-4} M. 2 : 4-D also stimulates growth, which then slowly returns to about the normal rate without showing any marked depression.

5. The possibility is suggested that IAA, which first accelerates elongation, is later converted into, or causes the production of, an inhibitor. When successive immersions in IAA are repeated every 2 hours, the inhibition can be partially overcome so long as the treatment is continued.

6. Ascorbic acid slightly depresses the rate of elongation, but triiodobenzoic acid and dichloranisole produce no significant effect.

7. Immersion of the horizontal rhizomes in IAA or 2 : 4-D causes them to turn up. This suggests that an equilibrium is disturbed and supports the hypothesis that the diageotropic behaviour of these rhizomes is the result of a balance between the effects of two opposing hormones.

INTRODUCTION

THE diageotropic position assumed by the rhizomes of *Aegopodium podagraria* s regarded by Bennet-Clark and Ball (1951) as the result of the action of two hormones working in opposition to one another, both being affected by gravity. Full verification of this hypothesis must await the isolation and identification of such hormones, but, in the meantime, it seemed that a study of the effects of known growth-regulating substances on these rhizomes was desirable as it might throw some further light on the mechanism concerned.

Journ. of Experimental Botany, Vol. 4, No. 12, pp. 349–62, Sept. 1953.

MATERIAL AND METHODS

Freshly dug portions of the rhizomes of *Aegopodium podagraria* about 12–18 cm. long were used. The growing-tips were photographed automatically every hour by infra-red radiation using the apparatus described by Bennet-Clark and Ball (1951), but with some minor modifications. To ensure that the rhizome chamber should be watertight, the front glass was clamped down on a rubber washer instead of merely being luted on to the metal frame with grease. In the earlier experiments the rhizomes were fixed in position by enclosing the basal regions between layers of wet filter-paper. As this arrangement would not have prevented movement when the chamber was flooded with water, a strip of sponge rubber was placed on top of the filter-paper and was pressed against it when the front glass was clamped down on the frame.

The rhizomes (20 in number) were placed in the chamber with their tips approximately horizontal and were left undisturbed for 24 hours to recover from the effects of light. Then the rubber tube used for passing moist air int the chamber was disconnected from the air supply and the experimenta solution was run in slowly until the chamber, which had a capacity of 1,60 ml., was completely full. In all cases the solutions were prepared with tap-water and were adjusted initially to pH 6·5 by addition of HCl, but no buffers were added. Forty-five minutes after the chamber was full the solution wa allowed to drain away and the chamber flushed out by filling it with water an again emptying it. The whole operation was carried out without exposing th rhizomes to light and was completed in the interval between two successiv photographs. These were taken at hourly intervals from the commencemen of the experiment and were continued usually until the end of the 71st hour Subsequently the photographic negatives were projected by an optical lanter on to a wall and the inclination of the tips to the vertical measured. The rat of elongation was calculated from other measurements of the projected images using a flexible scale to follow the curvature. Such measurements were mad at hourly intervals during a period immediately after the experimenta treatment; otherwise the increments after 3-hour intervals were recorded.

Nutational curvatures of the rhizome tips towards or away from the camer are an obvious source of error in estimates of length based on measurement of a photographic image. Also, unfortunately, rhizomes of *Aegopodium* are fa from uniform. Quite apart from other factors, the stage of development of th terminal internode may seriously affect its rate of elongation. In selectin rhizomes care was taken to choose those in which the terminal internode wa fairly short, about 5–25 mm., and therefore likely to be still in an active stat of elongation. Nevertheless, a small proportion showed negligible grow or, after a pause while the terminal bud swelled, produced a new internod which then began to elongate. The measurements of such aberrant rhizome were omitted in computing the means, but the number rejected did n exceed 3 out of 20 in any experiment and was usually less than 2. In spite variation in the material, replicate experiments with different batches izomes were reasonably consistent. Since this was the case, and because of e very large number of measurements involved, detailed statistical analysis as not attempted.

Room temperature varied from 15·3° to 21·3° C. during the course of the ork, but the range in each experiment was very much less and is recorded the text-figures.

EXPERIMENTAL RESULTS

ontrol experiments

In these experiments the treatment of the rhizomes was exactly as has been escribed, but water without the addition of any growth-regulating substance as used. The results of replicate experiments with different batches of hizomes are shown in Fig. 1. During the first 24 hours the growth rate rises. s will be seen later, this can be attributed to recovery from the effects of the ght necessarily received when the rhizomes are dug up and placed in the pparatus. Immersion in water for 45 minutes at the 24th hour produces no ignificant effect on the rate, although there are indications of a slight and emporary decrease followed by a small rise.

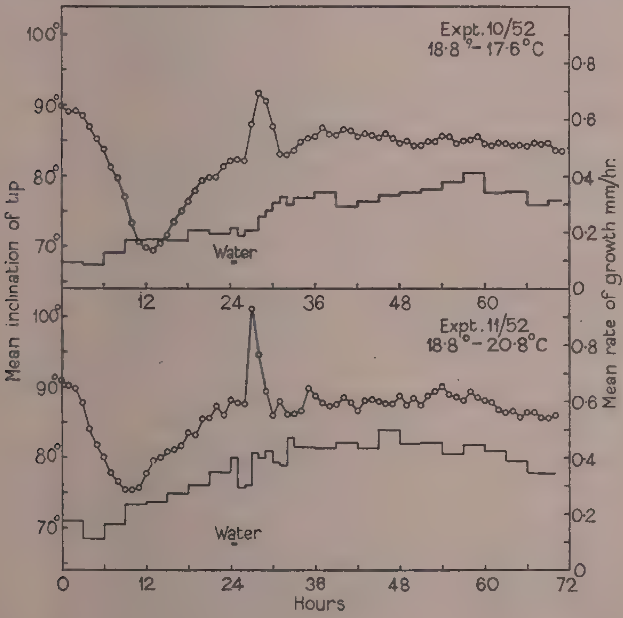

FIG. 1. Expts. 10/52 and 11/52. Effects on the growth rate (stepped curve) and on the inclination of the tips (circles) of the immersion of rhizomes for 45 minutes in water at 24th hour from the start of the experiment. The time of immersion and duration is indicated by the length of the thick black line.

As already pointed out by Bennet-Clark and Ball (1951), the tips of the hizomes bend down as a result of the illumination received while an experiment is being set up. The mean downward curvature due to the light reaches its maximum 10–12 hours later, and by the end of 24 hours has almost disappeared. Then, in these experiments in which the rhizomes were immersed in water for 45 minutes, a sharp upward curvature sets in about 1 hour after the end of the treatment. This comes to a maximum 1–2 hours later and then rapidly declines. The cause of this curvature, which for convenience will be referred to as the 'water effect', will be discussed later.

The effects of β-indolylacetic acid (IAA)

With the exception of certain preliminary experiments, the concentratio of IAA used was 10^{-4} M. The results of immersion for 45 minutes at the 24t

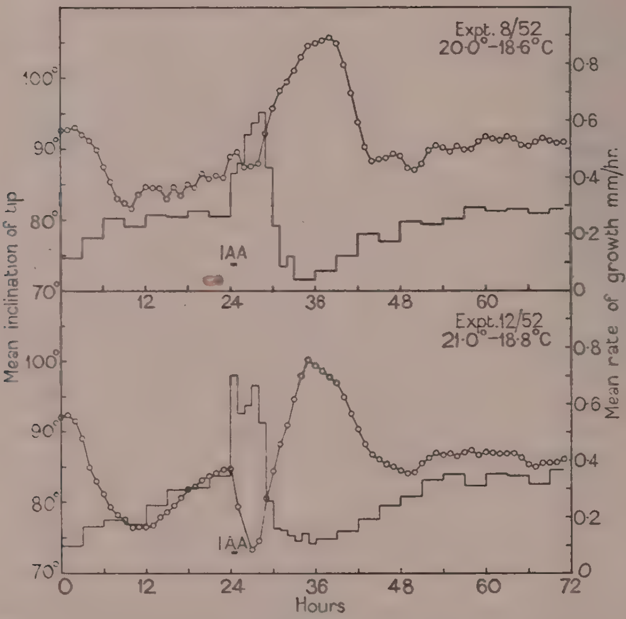

FIG. 2. Expts. 8/52 and 12/52. As in Fig. 1, but rhizomes immersed for 45 minutes in 10^{-4} M. IAA at the 24th hour.

hour are shown in Fig. 2. The growth rate rises rapidly, even while t rhizomes are still immersed, to about twice its former value. This increas rate is maintained for 5–6 hours. Then it falls very rapidly to a value whic considerably below what it was before the treatment. Subsequently, it sh a slow recovery.

It seemed at first that a possible explanation of the marked decrease in growth rate occurring after stimulation by IAA could be found in the t factor hypothesis put forward by Went (1928) and adopted by Dolk (19 to explain autotropism. This would suggest that the more rapid gro duced by IAA increases the rate at which some other essential substance is sed up, so that, when the concentration of this substance has been conderably reduced, the growth rate must fall and remain low until a further pply becomes available. One would expect, therefore, that a second treat-

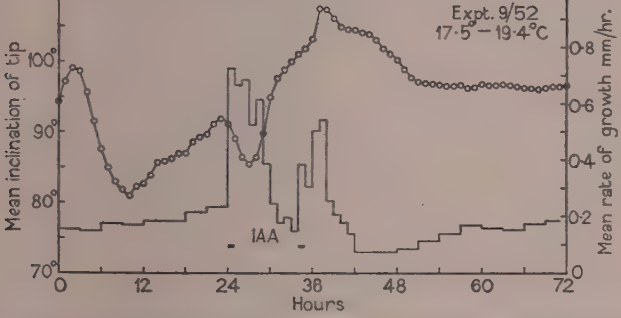

FIG. 3. Expt. 9/52. Effects of two immersions for 45 minutes in 10^{-4} M. IAA at the 24th and 34th hours. Times of immersion and durations are indicated by the lengths of the thick lines.

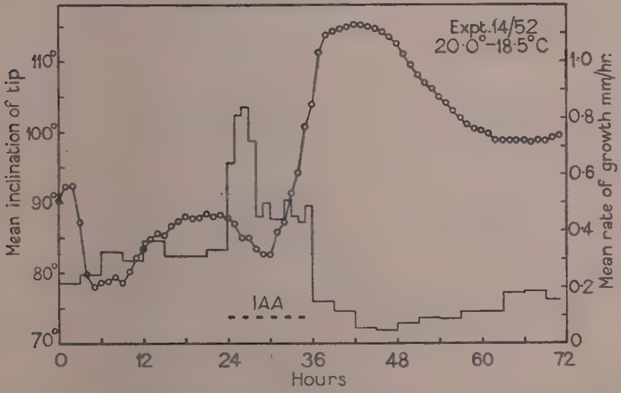

FIG. 4. Expt. 14/52. Effects of six immersions for 45 minutes each in 10^{-4} M. IAA, starting at the 24th hour and repeated every 2 hours. Other conventions as before.

ment with IAA after the growth rate had fallen would have little effect. Fig. 3 shows the result of such an experiment. Obviously a marked stimulation of growth by the second treatment with IAA has not been prevented by lack of some unknown substance.

Figs. 4 and 5 show the effects of 6 and 12 successive immersions in IAA for 45 minutes, the treatments being given every alternate hour. This seemed preferable to continuous immersion which might have interfered unduly with respiration. In both experiments the initial rise in the growth rate was succeeded a few hours later by a fall to a value about, or a little above, the normal. This rate remained fairly steady so long as the treatment with IAA was continued. As soon as this ceased, the rate fell sharply to a small fraction of its former value. The implication of these results will be discussed later.

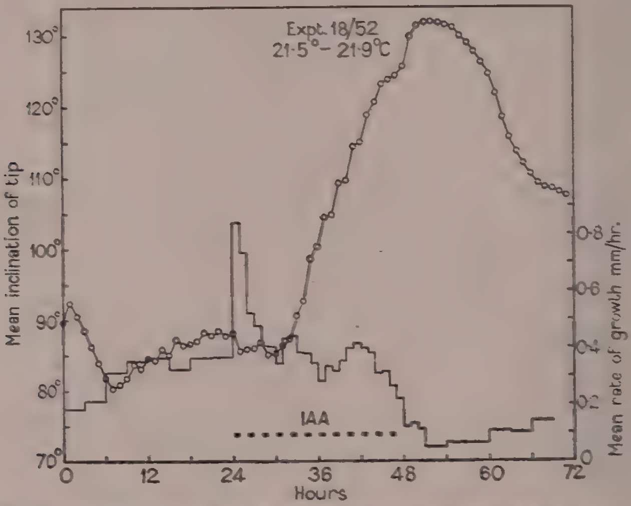

FIG. 5. Expt. 18/52. As in Fig. 4, but treatments continued for 24 hours.

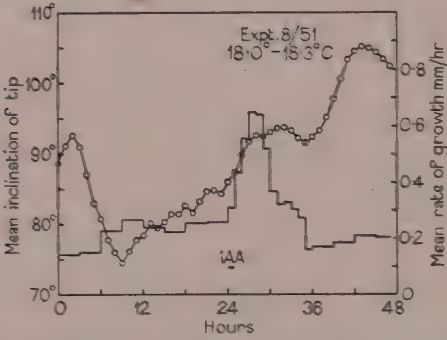

FIG. 6. Expt. 8/51. Effects of immersion for 45 minutes in 10^{-3} IAA at the 24th hour. This experiment was continued only until the end of the 47th hour.

The effects of IAA on the inclination of the tips is shown in the graphs During the period of maximum growth there is a general tendency for th rhizomes to curve down. Then, as the growth rate declines, this tendency i reversed and they curve up. Where the immersions are repeated (Figs. . and 5), this upward curvature continues until the treatments are concluded Then it ceases and is gradually reversed. The cause of these various movement will be discussed later.

In some earlier experiments a more concentrated solution of IAA, 10^{-} M., was used. The results of one of these is shown in Fig. 6. The effect o ne growth is not very different from that produced by a 10^{-4} M. solution, ut the changes in the inclination of the rhizomes were very erratic. Some hizomes began to bend up as the growth rate declined, while others did not o so until much later. The cause of this variation in behaviour is not yet lear, but that acceleration of growth after immersion in 10^{-3} M. IAA does ccur was confirmed in two other experiments in which the photographic ecording was unfortunately incomplete.

ffects of 2:4-dichlorophenoxyacetic acid (*2:4-D*)

Fig. 7 shows the results of replicate experiments in which the rhizomes were reated with 10^{-4} M. 2:4-D instead of IAA. As before, the growth rate goes up rapidly and then, after 5–6 hours, declines. In this case the decline is much more gradual than with IAA and the rate shows no significant tendency to sink below what it was before the treatment, although from about 42 to 48 hours it is slightly lower than in the control experiments. Fig. 8 shows the effects of two treatments with an interval of 10 hours. The second one also pushes up the growth rate and then this slowly reverts to about the normal value.

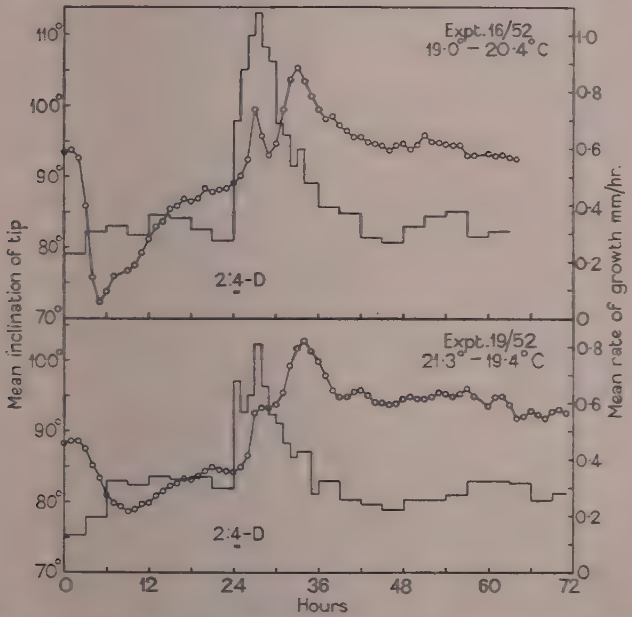

FIG. 7. Expts. 16/52 and 19/52. As in Fig. 1, but rhizomes immersed for 45 minutes in 10^{-4} M. 2:4-D at the 24th hour. In Expt. 16/52 some of the rhizomes had grown beyond the photographic field of view by about the 63rd hour so that further measurements became impossible.

The effects of 2:4-D on the inclination of the tip is clearly different from that of IAA, at any rate with the concentrations which were used. Very shortly after the immersions the rhizomes tend to curve up and then down, just as they did in the control experiments with water. This 'water effect', which was absent in the IAA experiments, is followed and may be partially masked by an upward curvature due to the 2:4-D. As with IAA, this upward curvature develops as the growth rate is declining. In Fig. 8 the curve shows a series of four peaks. From their positions along the time axis it is probabl that the first and third are due to the 'water effect', and that the second an fourth are caused by 2:4-D.

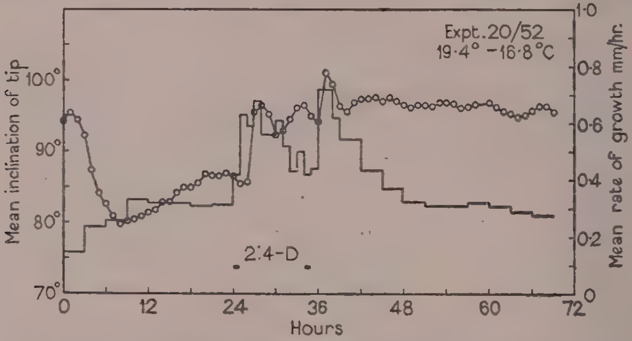

FIG. 8. Expt. 20/52. Effects of two immersions for 45 minutes in 10^{-4} M. 2:4-D at the 24th and 34th hours. The drop in the growth rate during the last 12 hours was probably due to the fall in temperature.

Effects of possible growth inhibitors

Both IAA and 2:4-D cause an upward movement of the rhizome tips Presumably they become more concentrated on the lower side of the rhizom and disturb the equilibrium between opposing hormones which determine the horizontal position. It would seem, therefore, that treatment of th rhizome with a growth inhibitor might have the opposite effect and cause th tips to turn down. The results of experiments with a limited range of possibl inhibitors are shown in Fig. 9. 2,3,5-triiodobenzoic acid (TIBA) was considered by Galston (1947) to antagonize the action of IAA. 2:4-dichloranisol (DCA) was found by Bonner (1949) to inhibit the growth of the *Aven* coleoptile, although Audus and Shipton (1952) describe experiments whic make it seem unlikely that DCA can be regarded as an effective anti-auxi Ascorbic acid was used on the suggestion of Professor T. A. Bennet-Clar who found (private communication) that it decreased the elongation of coleo tile sections when treated with IAA.

TIBA, 200 mg./l., and DCA, 10 mg./l., produced no significant effect the growth rate. Ascorbic acid, 0·1 per cent., gave a slight depression. Con bining the two experiments, comparison of the elongation of 37 rhizom during the 3 hours after treatment with ascorbic acid with that during t 3 hours before shows a drop of 17·0±6·9 per cent., which is significant.

evidence of any effect of these substances on the inclination of the tips can be detected, but all the curves show an up-and-down movement—the water effect—similar to that which occurred in the control experiments (Fig. 1) using water.

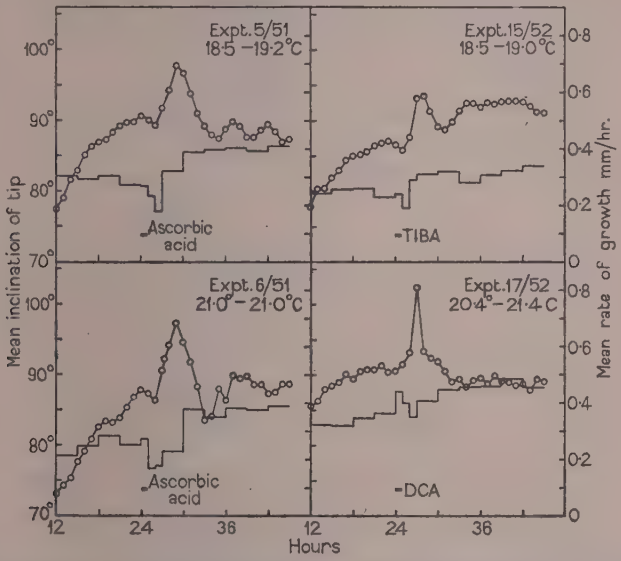

FIG. 9. Expts. 5/51 and 6/51. Effects of immersion for 45 minutes in 0·1 per cent. ascorbic acid at 24th hour. Expts. 15/52 and 17/52. Effects of similar immersions in (top right) triiodobenzoic acid, 200 mg./l., and (bottom right) dichloranisole, 10 mg./l. In order to save space only the portions of the curves between the 12th and 45th hours are shown.

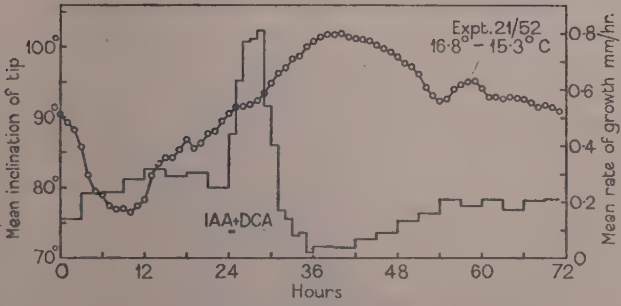

FIG. 10. Expt. 21/52. Effects of immersion for 45 minutes in 10^{-4} M. IAA + 10 mg./l. DCA at the 24th hour.

In another experiment 10 mg./l. DCA was added to 10^{-4} M. IAA. The results with this solution are shown in Fig. 10. They are very similar to those obtained with IAA only (Fig. 2). There is no decrease in the stimulation due to IAA. The subsequent retardation is certainly very marked. Many of the rhizomes stopped elongating for a time, but the effect is not significantly greater than that obtained with IAA alone.

Effect of light. The effect of light on the inclination of the rhizomes was described by Bennet-Clark and Ball (1951), but was not studied in relation to the general effect of light on the growth rate. The results of such an investigation are shown in Fig. 11. Twenty-four and a half hours after the start of the experiment, one of the infra-red filters was removed temporarily and th rhizome tips exposed for 5 minutes to the 60-watt strip light at a distance o 28 cm. Then the filter was replaced and hourly recording by infra-red photography continued as before. Immediately after the illumination there was sharp drop in the growth rate. In 2 hours this had diminished to about hal its former value. Then there was a gradual recovery extending over about 2 hours. These observations suggest a rapid inactivation of auxin in the light followed by a slow replacement or re-activation in the dark. The slow recover explains the rise in the growth rate shown in the present series of experiment during the first 24 hours after the rhizomes were enclosed in the rhizom chamber and placed in the dark.

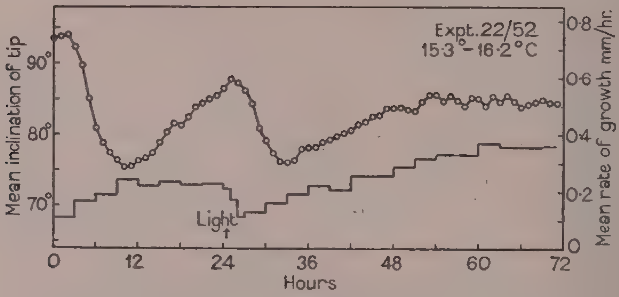

FIG. 11. Expt. 22/52. Effects of a light stimulus for 5 minutes between the 24th and 25th hours.

The effect on the inclination of the tips also is shown in Fig. 11. The downward curvature and subsequent recovery after exposure to light can b explained in terms of a general decrease in the amount of an auxin, provide that the opposing hormone is less sensitive to light. The effect of ligh appears to be to disturb the equilibrium between the two hormones, leavin an excess of anti-auxin, thus promoting the downward curvature. As th auxin gradually returns to its original concentration, the equilibrium restored.

DISCUSSION

One interesting result of these experiments is the marked difference betwee the effects of 10^{-4} M. solutions of 2:4-D and IAA on the growth in lengt of the rhizomes of *Aegopodium*. Both of these substances cause a rapid an marked acceleration which persists for several hours after the treatment an then declines. With 2:4-D the decline is gradual as the substance diffus way or is destroyed, and the growth rate returns to about what it was before. fter IAA, on the other hand, the drop is much steeper and the growth rate nks to one-third or less of its original value.

Certain suggestions can be made to explain this very definite depression in he growth rate. The first, which has already been mentioned, is the exhaustion f some essential food factor as a result of the increased rate of growth. This xplanation may be rejected since a second treatment with IAA again produces high rate of growth and since the increase of growth caused by 2:4-D is not ollowed by any marked inhibition. The depression in the growth rate which ollows the acceleration due to IAA must therefore be the result of treatment with this substance and not merely a consequence of the increased rate of growth.

The actual depression in the growth rate may be due to a decrease in the ensitivity of the tissues, to the conversion of IAA into an inhibitor, or to he production of an inhibitor from some other source as a result of the action of IAA. There is no clear evidence from these experiments to aid one to decide between these alternatives, and until further work has been done speculation would be unprofitable.

The inhibitory effect which develops some hours after the rhizomes are reated with IAA is not a direct consequence of the rather high concentration used, since it can be overcome to a considerable extent by further treatment with the same substance, as seen in Figs. 3, 4, and 5. Where successive reatments are given during 12 or 24 hours the rate of growth becomes fairly steady. If an inhibitor is produced, then presumably the rates of formation and decline of the postulated inhibitor would tend to become equal and a balance be reached between its effect and that of the entering IAA. Very soon after the immersions in IAA are discontinued its concentration becomes insufficient to counteract the inhibitor and the rate of growth rapidly declines.

The form of the growth curves derived from these experiments serves to emphasize a point made recently by Audus (1952) that in studying the effect of chemical agents on growth, the magnitude and time relationships of the growth curves are of more fundamental importance than a measurement of total growth after an arbitrary interval of time. That the choice of this interval of time may profoundly affect the results is demonstrated in the following table which is based on the results shown in Figs. 1, 2, and 7. The effects of various treatments on the mean growth rate of the rhizomes are given, together with the standard errors, for the periods 24–30 hours and 24–48 hours. In each case the rate is shown as a percentage of the mean growth rate of that experiment during the period 18–24 hours, that is, immediately before the treatment was applied.

It is obvious that if conclusions had been based on comparisons of the mean growth rate of the rhizomes during the whole 24-hour period after treatment, the completely false inference would have been drawn that the effect of 10^{-4} M. 2:4-D in promoting elongation is only slightly greater than that of water and that 10^{-4} M. IAA acts only as an inhibitor.

No. of rhizomes	Treatment 45 min. in	Growth rate (percentage of pre-treatment rate) 18–24 hours	24–30 hours	24–48 hours
36	water	100	113·2±5·6	132·2±3·7
38	IAA	100	179·8±7·1	81·5±4·7
37	2:4-D	100	252·1±13·6	151·4±6·6

Many investigations of the effects of growth substances on plant organs have been based on measurements of total elongation, one of the reasons being that a series of measurements of length cannot easily be made withou exposing the tissues to light. Apart from infra-red photography, other ways o meeting this difficulty are available. Audus (1952) in studying the effect of th time factor on the arsenate inhibition of growth of *Pisum sativum* estimate the growth by a series of measurements of weight, using red light for sten sections and diffuse white light for sections of the root. Earlier, Laibach an Kornmann (1933) used an interferometer method to measure the rate o growth of a decapitated coleoptile while it was surrounded by water and the while it was immersed in an aqueous extract of orchid pollinia. They foun that during the first 2 hours after the extract was applied the growth rate ros to about 3 times its former value. Then it declined and became approximatel steady at about twice the original rate. The general form of the growth curv they give is rather like that obtained with *Aegopodium* rhizomes while subjecte to six successive immersions in IAA (Fig. 4), but, since in Laibach and Kornmann's experiment the effect of discontinuing the treatment with growt substance was not investigated, it is not known whether this also would hav resulted in the growth rate falling to less than its original value.

Turning now to the effects of the various treatments on the inclination the tips of the rhizomes to the direction of gravity, the first point to be considered is what has been described as the 'water effect'. This is the shar upward curvature which commences about 1 hour after immersion. It reache a maximum a few hours later and then is reversed. This movement occurre in all the experiments except in those in which IAA was applied. It develope in the control experiments without added growth substance and occurre after immersion in 2:4-D during the period of vigorous growth when presumably the concentration of growth-promoting substance was not limitin Therefore it seems unlikely that the movement is caused by differences in auxi concentration on the two sides. The actual cause of the movement is not know but it agrees very closely in magnitude and timing with a similar up-and-dow curvature described by Bennet-Clark and Ball (1951) when the rhizomes we treated with 5 per cent. carbon dioxide. It is possible, therefore, that it is d to some effect of immersion on gaseous exchange. Another possibility is th after immersion the growing region remains wet, and that then the adheri water drains to the lower side where it remains for a longer time and th facilitates the expansion of the cells on that side, causing upward curvatur

Another problem is to explain why the water effect should occur aft immersion in 2:4-D but not IAA, although in both cases the rhizome chamber was flushed out with water after treatment. During the period when the water effect was evident in other experiments, rhizomes treated with IAA showed either no significant curvature or else a very marked downward bending (Figs. 2, 3, and 4). Evidently the tendency to curve down suppresses or completely reverses the water effect. Unlike the downward bending, which occurs after illumination and has been attributed to destruction of auxin by light, this downward movement occurs while growth is extremely vigorous and while IAA in the tissues is presumably present in excess. The reason for it is still unknown.

Apart from the upward curvature due to the water effect, or the downward movement which may replace it after treatment with IAA, it is evident from the graphs that both IAA and 2:4-D bring about a definite upward curvature, although the movement does not commence until the growth rate has begun to decline. For some hours after immersion in the solution the concentration of growth-promoting substance in the tissues is probably in excess and therefore small differences in concentration on the two sides would be unlikely to have any effect. But, as the total concentration declines, a point is reached where the rate will begin to depend on the concentration and then any accumulation of auxin on the lower side will become effective and produce upward bending. Where repeated immersions in IAA were carried out (Figs. 4 and 5) the upward curvature commenced soon after the growth rate had fallen to its equilibrium value. This may indicate that, although IAA is still being supplied as before, the effective concentration in the tissues has become sub-optimal and supports the suggestion that an inhibitor has been formed. The upward curvature continues to increase so long as IAA is supplied, and where this was done for 24 hours some of the rhizomes finally pointed almost vertically upwards. With the fall in the growth rate the upward movement ceased and then, as the rate began to rise again, the rhizomes bent down (Figs. 2, 4, 5, and 10).

If the assumption is correct that an inhibitor, and not a decline in sensitivity, is produced in the tissues by IAA, then more of this inhibitor will develop on the lower side of the rhizome where the hormone has accumulated. Where the treatment is continued for 12 or 24 hours one may postulate that the entering IAA tends to become more concentrated on the lower side and overcomes the inhibitor, but that when the treatment has stopped the excess inhibitor on the lower side begins to exert its effect and the rhizomes bend down. Other factors, such as those suggested by Bennet-Clark and Ball (1951) to explain the normal diageotropic response, may co-operate, but it is noticeable (Figs. 2 and 7) that recovery is less complete after treatment with 2:4-D, which does not produce any marked inhibitory effect, than it is after IAA.

CONCLUSION

As mentioned in the introduction, this investigation was undertaken in the hope that it would throw some further light on the diageotropic response.

Other points of interest have emerged, but support is found for the hypothesis advocated by Bennet-Clark and Ball (1951), that the horizontal position of the rhizomes is due to an equilibrium between the effects of opposing hormones, by the fact that immersion of the rhizomes in solutions of IAA or 2:4-D makes them not only grow faster but also curve upwards. There must therefore be a mechanism causing these substances to accumulate on the lower side. Since the same may be assumed to happen to any auxin produced in the rhizome itself, the conclusion seems to be unavoidable that the horizontal position can result only from the balancing of the excess hormone on the lower side by an opposing hormone. This view receives further support from comparison of the effects of light on the inclination and growth rate of the rhizomes.

The suggestion that β-indolylacetic acid not only causes an acceleration of growth, but subsequently produces an inhibitory effect, and possibly may itself be converted into an inhibitor, obviously needs further investigation, not only in *Aegopodium* rhizomes but also in other plants. If it should prove to be generally valid, it would provide a simple and satisfactory explanation of autotropism. Further experiments are planned, but unfortunately the progress of work on these rhizomes must inevitably be slow owing to the special arrangements necessary because of their sensitivity to light and to the fact that experiments can only be carried out during the growing season. Direct evidence of the nature of the growth substances present in *Aegopodium* may also be expected from chromatographic analyses which are being carried out independently by other workers in this department.

I wish to thank Professor T. A. Bennet-Clark for providing facilities for this work and for continued encouragement and advice.

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The Use of Tracheal Sap in the Study of Apple-tree Nutrition

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SUMMARY

A method of extraction of tracheal sap from shoots of apple-trees in quantities sufficient for analysis is described. Total solids, conductivity, nitrogen, phosphate, potassium, and magnesium levels were determined in saps from trees of a single variety on different stocks through one season. A marked seasonal fluctuation in levels of each of the four elements was noted.

INTRODUCTION

MOST apple-trees consist of two varieties, one forming the stock and another he scion, both clonal in origin. Any study of tree nutrition becomes compliated when the numerous stock/scion combinations are considered. For nstance, Wallace (1950) has pointed out that some apple varieties used as cions have a high potassium and others a high magnesium requirement, and hat the intensity of potassium or magnesium deficiency symptoms may also vary according to stock variety.

Wormall (1924), Priestley and Wormall (1925), and Burstrom and Krogh (1946, 1947) have studied bleeding sap composition. Dixon and Atkins (1915, 1916) extracted sap by centrifuging short lengths of freshly cut wood. Bennett, Anderssen, and Milad (1927) showed that gas displacement was a more satisfactory method of obtaining tracheal sap. Using this technique Anderssen (1929), Oserkowsky (1932), and Bennett and Oserkowsky (1933) made conductivity measurements and determined the iron and copper content of tracheal saps of a range of fruit-trees.

In the work reported here the possibility of using such an extraction technique for studying the nutrition of apple-trees has been investigated.

MATERIALS AND METHODS

Trees. The trees used were 9-year-old apples, variety 'Granny Smith', worked on three rootstocks—'Northern Spy', 'Malling XII', and 'Malling XVI'—and growing at the Oratia Research Orchard on a heavy clay soil (pH 5·4). To provide a supply of the previous season's shoots at the beginning of the 1951–2 season, no pruning was done in the winter of 1951. On 4 October 1951 each tree received 5 lb. of a manurial mixture made up of 4 parts superphosphate, 4 parts blood and bone, and 1 part sulphate of potash.

Through the season the trees showed the following development: 10 September 1951, slight bud swelling; 17 September 1951, early green tip;

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24 September 1951, green tip; 1 October 1951, open cluster—pink; 8 October 1951, pink—full blossom; 15 October 1951, full blossom; 23 October 1951, petal fall; 1 May 1952, fruit picked; 9 June 1952, first leaves falling; 7 July 1952, most of leaves off the tree.

Selection of shoots. Shoots, approximately 100 cm. long with a base diameter of 1 cm., were cut from the trees, only vigorous growths from the ends of lateral branches being selected. To prevent evaporation any leaves present were removed at time of sampling. Shoots were gathered between 8 and 9 a.m. At each sampling two or three shoots were taken from each of nine trees (three on each rootstock), and from each tree one sap sample was obtained by combining the saps extracted from the several shoots. In all, thirty-one samplings were made through the season at varying intervals. From August to December samples were taken weekly, from December to March every 2 weeks, and from March to August the intervals were lengthened to 3 or 4 weeks.

Extraction of sap. Sap was extracted by a simplification of the gas displacement method of Bennett *et al.* (1927). Shoots were stripped of bark for 5 cm. from the base and set in a rubber bung in a 500-ml. filtering flask. A test-tube (150 × 15 mm.), supported on cotton-wool inside the flask, was set under the proximal end of the shoot. Sap was extracted by reducing pressure inside the flask to a vacuum of 24 in. of mercury and then cutting successive sections off the distal end of the shoot. Cutting was continued until each shoot was cut down to the insertion in the rubber bung. By removing a section about 0·5 cm. long every 2–3 seconds, a steady flow of sap was maintained. Each shoot yielded, on the average, 3·5 ml. of sap. Sap samples thus obtained were immediately filtered through paper, as samples went brown unless bark and wood fragments were removed, and if kept for any later determinations were always frozen. This technique extracted from the shoots, at any stage of growth, 20–25 per cent. of the total moisture determined by oven drying at 105° C.

Sap analysis. Six determinations were made on each sap sample collected viz. total solids, conductivity, nitrogen, phosphate, potassium, and magnesium.

Total solids were estimated by difference in weight when 1 ml. of sap wa evaporated to dryness in a 3-ml. phial in a desiccator at room temperatur under reduced pressure and over sulphuric acid. Results are expressed a mg. per ml. of sap.

Conductivity values were obtained by measuring the electrical resistanc of the sap using an A.C. electronic bridge and 1-ml. conductivity cell. Sa conductivities are expressed in terms of normality of standard potassiun chloride solutions determined at the same time.

Total nitrogen. As no nitrate could be detected with phenoldisulphoni acid, no precautions were taken to include it in the total nitrogen. Results ar expressed μg. N/ml. sap.

Phosphate, potassium, and magnesium. Colorimetric methods used b icholas (1948) were reduced in scale for these determinations. Volumes of ss than 0·2 ml. were measured using Kimble 0·2-ml. pipettes. Colours were veloped in 3-ml. phials and measured in a Spekker Absorptiometer using icro (0·5 ml.) cells.

RESULTS

Nature of the sap. The sap at all seasons was a clear, colourless liquid of H 4·9–5·3. It could be extracted from shoots in comparable quantities roughout the year. From the following considerations it is believed that the p obtained by this technique is that present in the cavities of the xylem ssels and is not seriously contaminated by the contents of living cells. omparable quantities of sap were obtained from whole shoots and from ose from which all the bark (i.e. all tissues external to the wood) had been ripped before extraction. Sap of similar nutrient concentration was obtained om two halves of the one shoot, when one half had had bark stripped off efore extraction. An alcoholic extract of wood tissues contained different lative amounts of nitrogenous compounds from the sap.[1] Sugars were bundantly present in the alcoholic extract, while they were nearly always bsent from the sap.[1]

Composition of the sap. The total solids, conductivity, phosphate, potassium, nd magnesium contents of each of the sap samples on each of the sampling ates are represented graphically in Figs. 1–6 respectively. Results for trees n the three rootstocks are given separately and each point on the graphs epresents a mean value for three trees. The vertical lines in each figure epresent the differences necessary for significance at 5 per cent. level between he means for different sampling times within stocks.

Each of the four elements measured could be detected at almost all times n the sap, but there were definite seasonal fluctuations. After a period of low nd fairly constant levels during the winter, the period of flower opening was narked by a rapid rise in sap concentration. Within 14 days there was a seven-old increase in nitrogen and a five-fold increase in phosphate and magnesium. There was also a seven-fold increase in potassium, but this was more gradual hen with the other elements, reaching peak values 6 weeks later. Except for a minor peak in mid-December there was then a gradual decrease in concentration of all elements until, by harvest time, each element had reached its low winter level. The levels of total solids and electrolytes (as measured by conductivity) showed the same pattern of seasonal trend. The fluctuation in sap concentration was not found to be correlated with rainfall in the week preceding sampling.

In general, the fluctuations of each of the elements in saps from the different rootstocks followed each other closely. Nevertheless, there were instances where significant differences between stocks did occur. Over the period 1–23 October (blossoming) sap from trees on 'Malling XVI' contained significantly (at 1 per cent. level) more nitrogen than trees on the other stocks. Over part of

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[1] Detailed methods and results will be published separately.

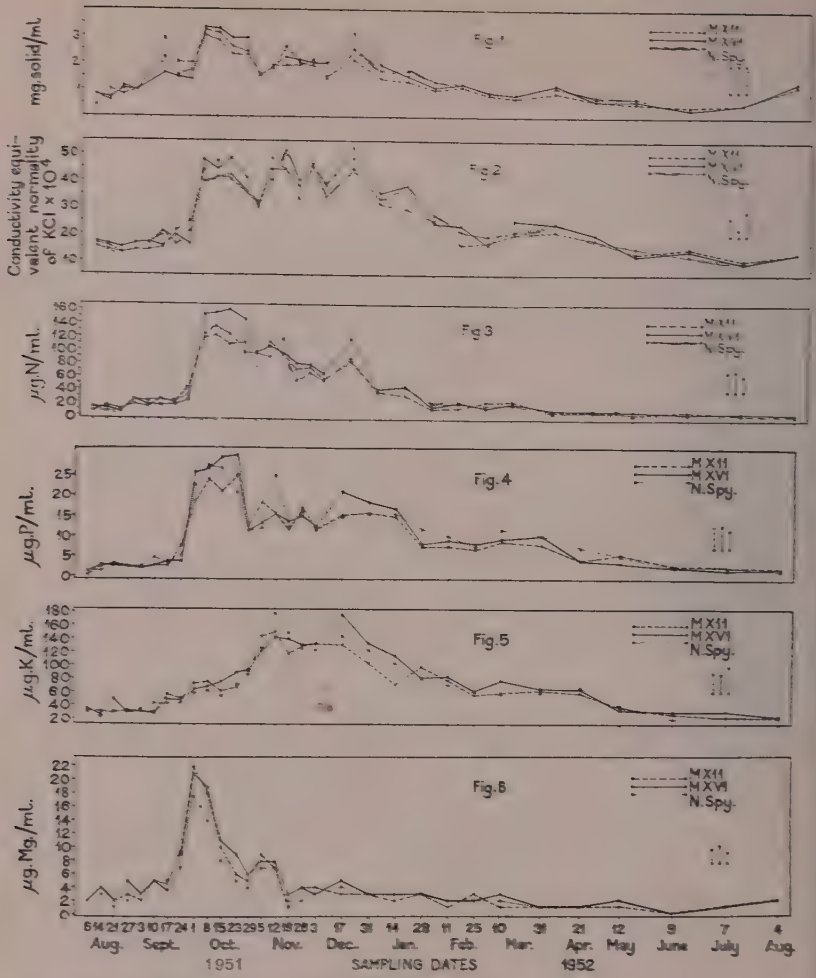

FIG. 1. Seasonal variation in weight of solids in tracheal sap of 'Granny Smith' apples on three stocks (mg. per ml. of sap). FIG. 2. Seasonal variation in conductivity of tracheal sap of 'Granny Smith' apples on three stocks, expressed as equivalent normality of KCl × 10^4. FIG. 3. Seasonal variation in total nitrogen of tracheal sap of 'Granny Smith' apples on three stocks (μg. per ml. of sap). FIG. 4. Seasonal variation in phosphate of tracheal sap of 'Granny Smith' apples on three stocks (μg. per ml. of sap). FIG. 5. Seasonal variation in potassium of tracheal sap of 'Granny Smith' apples on three stocks (μg. per ml. of sap). FIG. 6. Seasonal variation in magnesium of tracheal sap of 'Granny Smith' apples on three stocks (μg. per ml. of sap).

the same period, the nitrogen level was significantly higher (at 1 per cent.) for 'Northern Spy' than for 'Malling XII'. The phosphate content of sap from trees on 'Malling XVI' was significantly (at 1 per cent.) higher than from trees on the other two stocks over the period 17 December to 14 January. Over this same period the potassium level was higher in 'Malling XVI' than in 'Northern

Spy', which was higher than in 'Malling XII', both differences being significant at 1 per cent. The magnesium level for sap from trees on 'Northern Spy' was less (at 1 per cent.) than from the other stocks from 1 to 29 October. On 12 November values for all determinations (except solids and magnesium) on sap from 'Northern Spy' were significantly (at 1 per cent.) higher than from the other two stocks. On 17 December values from 'Malling XVI' were higher (at 1 per cent.) than from 'Malling XII' for all determinations, and higher (at 1 per cent.) than from 'Northern Spy' for all except solids and magnesium.

DISCUSSION

The method of sap extraction described provides a ready means of obtaining tracheal sap from apple shoots at all times of the year in quantities sufficient for analysis of major constituents. Sap so extracted has the advantage of not having been subjected to heat or any chemical extractants. For the reasons adduced, it seems that the liquid obtained is largely a sample of the transpiration stream from the cavities of xylem vessels with little, if any, contamination resulting from collapse of living cells of xylem or external tissues. The method should be applicable to many woody plants. A disadvantage is that there is a minimum size for shoots to yield sap in workable quantities.

Work with radioactive tracers (Stout and Hoagland, 1939) has confirmed the older view (Mason and Phillis, 1937) that elements absorbed by roots travel upwards in the transpiration stream, and has also shown that there is a continuous lateral movement of nutrients from xylem sap to bark tissues. Biddulph (1951) suggests that there are losses of materials from the transpiration stream directly to the cambium and young phloem and through the rays to other tissues. While it seems reasonable to believe that the liquid studied in the present work originally contained most of the nutrients absorbed from the soil, some losses to other tissues will probably have occurred before it reaches the young shoots which were sampled, e.g. it will have passed fruit-bearing parts of the tree.

Analyses measured concentration of the sap at time of sampling only, and gave no measure of total amount of nutrients passing up from the roots, this being a function of rate of flow as well as sap concentration. Maximum concentration of most elements occurred before the trees were in full leaf, but it is possible that greater amounts of nutrients passed to the scion at times when the sap concentration was not at its greatest, but transpiration had increased.

Seasonal fluctuations are marked, particularly the rise in concentration of nitrogen, phosphate, and magnesium in 1 week at blossom time. Some of this sudden increase in sap concentration may be due to mobilization of reserves rather than increased nutrient intake by the roots. It is not apparent why potassium should show a different pattern from the other elements.

Though the changes in nutrient concentration are very similar throughout for trees on different rootstocks, there were still, at times, significant differences for each of the elements. It is possible that these differences may reflect some of the nutritional characteristics of the stocks.

This technique should allow comparisons to be made between trees under different treatments. In any such comparative work it would be preferable, where possible, to sample before nutrient levels become too low; with apples under the conditions of the present work this is before the end of January.

ACKNOWLEDGEMENTS

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The Effect of Aeration on the Metabolism of Acetate by *Aspergillus niger*

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SUMMARY

1. The effect of passing different rates of air or graded concentrations of CO_2 over cultures of *A. niger* growing on an acetate medium has been investigated.

2. There is a rate of aeration or concentration of CO_2 which is optimal for most rapid utilization of acetate. High rates of air-flow severely depress consumption of acetate, whereas very slow rates only slightly increase the metabolic period.

3. Production of oxalate is not greatly affected by aeration or by different concentrations of CO_2 except where consumption of acetate is reduced.

4. Citrate formation is markedly affected by the experimental conditions. Under optimal treatment citric acid yield is increased by amounts up to 64 per cent. with controlled aeration and by amounts up to a further 180 per cent. with regulated concentration of CO_2.

INTRODUCTION

THE ability of *A. niger* to produce citric and oxalic acids from acetate is well known, although the relationship of the mechanisms concerned to the tricarboxylic acid cycle is still uncertain (Walker, 1949; Foster, 1949; Foster and Carson, 1950; Lewis and Weinhouse, 1951). The present work was undertaken in order to investigate this problem further. In particular it was desired to determine whether the alteration of oxygen supply and the imposition of a back pressure of CO_2 would reduce the combustion of acetate and favour the production of less oxidized compounds, such as citrate, instead of oxalate and CO_2. Furthermore with aeration restricted, carboxylation reactions might be expected to occur more readily leading to interference with the tricarboxylic acid system, should the latter proceed in the mould. Most carboxylative processes, however, require energy and therefore the final outcome of metabolism would be determined by the balance between exergonic reactions such as oxidation of acetate and endergonic reactions such as carboxylations. Chugtai and Walker (1951) have not only shown that arsenite inhibited decarboxylative reactions in *A. niger* but also that it increased the yield of citrate from glucose.

EXPERIMENTAL METHODS

Cultures

The strain of *A. niger* NI as used in the past by Walker (1949) was employed as surface cultures. Two types of medium (I and II) were prepared

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based on that of Currie (1917), both with acetate in place of sugar, while ammonium chloride (0·066 g./100 ml.) replaced ammonium nitrate in medium II. The composition of medium I was NH_4NO_3 0·200 g./100 ml.; KH_2PO_4 0·075 g./100 ml.; $MgSO_47H_2O$ 0·021 g./100 ml.; HCl(11·3N.) 0·100 ml./100 ml.; acetic acid 3·000 g./100 ml.

Medium II is more conducive to citrate formation from acetate by *A. niger* than is a medium containing ammonium nitrate. The carbon source was incorporated before adjusting the pH to about 6·9 with sodium hydroxide. Cultures were grown in 2·4-litre flasks with 500 ml. of medium. These vessels were assembled for controlled aeration and for gas sampling according to Hockenhull (1946). The medium was sown with 1 ml./50 ml. medium of a spore suspension containing *c.* 10^9 spores/ml. in 0·1 per cent. (v/v) calsolene oil HS (I.C.I. Ltd.) prepared from slopes on glucose-agar which had been incubated for 10 days. All cultures were grown in the dark at 30° C.

Analytical techniques

Acetic acid. The culture filtrate was distilled in the manner previously reported (Halliwell and Walker, 1952) from sodium tungstate, sulphuric acid, and magnesium sulphate after Friedemann (1938). The distillate was titrated against phenolphthalein using 0·025 N. sodium hydroxide in a microburette. *Oxalic acid.* This was determined by precipitation of the calcium salt and titration with permanganate (Halliwell, 1950). *Citric acid.* The pentabromoacetone procedure of Taussky and Shorr (1947) proved to be the most suitable of several investigated. *Gas analysis.* The Haldane apparatus was used throughout. *Chromatographic analysis.* The paper procedures of Lugg and Overell (1947, 1948) for carboxylic acids and of Cavallini, Frontali, and Toschi (1949) for aldehydes, ketones, and keto-acids were employed.

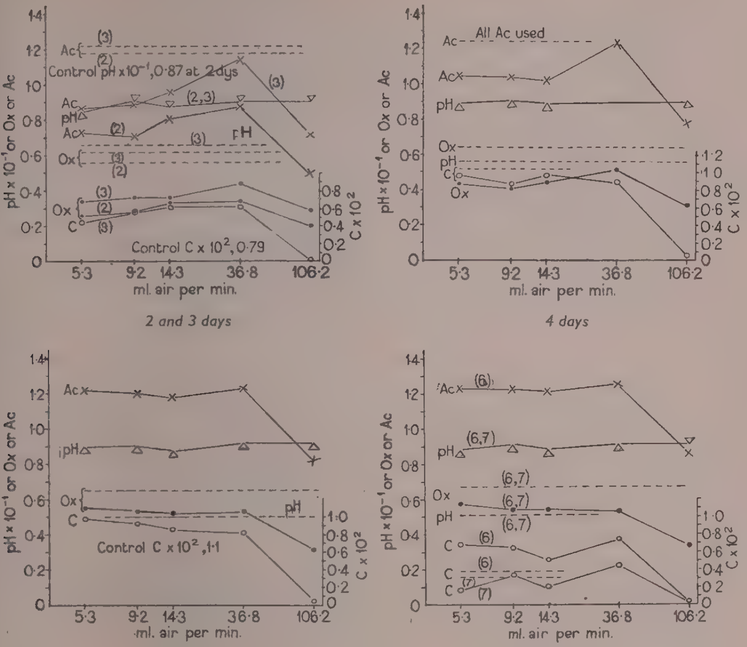
EXPERIMENTAL RESULTS

A. *The effect on acetate metabolism of renewal of the atmosphere above the cultures*

Expt. 1. Medium I. The cultures were allowed to grow with free diffusion of air through cotton-wool plugs for about 4 days so that thin growth was established. They were then subjected to aeration at rates varying from 5 ml. to 100 ml. air/minute (0·01 ml. to 0·20 ml. air/ml. medium/minute). Small samples were withdrawn periodically for analysis, the total withdrawals being less than 15 per cent. of the initial volume. The results of Expt. 1 are shown in Fig. 1.

Acetate disappearance was optimal at about 36 ml. air/min. when, for example, 91 per cent. of the available acetate had been consumed in 3 days' aeration; the corresponding figures at the same period for the lowest and highest air rates were 70 and 58 per cent. respectively. The optimal rate of aeration corresponded closely to that achieved under free diffusion of air through a cotton-wool plug (control). A flow-rate as small as 5 ml. air/min.

increased the time required for complete utilization of acetate by only 1 or 2 days.



2 and 3 days

4 days

5 days

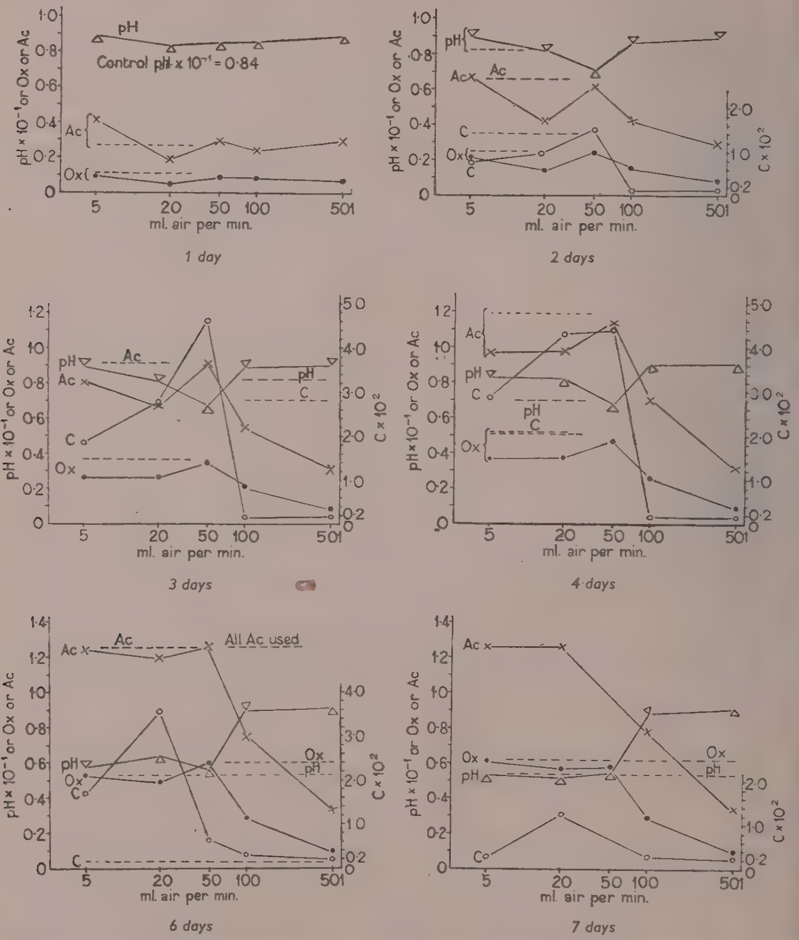
6 and 7 days

FIG. 1. Metabolism of acetate in Expt. 1 during 7 days' aeration. Aeration rates in ml. air/min./culture on log scale are shown as abscissae. Ordinates represent pH $\times 10^{-1}$ acetic acid (*Ac*) used, and oxalic (*Ox*) and citric (*C*) acids produced. Values for *Ac* and *Ox* are given in g. carbon/100 ml. medium, and for *C* in g. carbon $\times 10^{2}$/100 ml. medium. Symbols: pH–△–; *Ac* ·× –; *Ox* ·•–; *C*–○ -. Control values indicated by pH, *Ac*, *Ox*, or *C* beside a broken line (- - - -) parallel to abscissae, or alternatively, where confusion would have arisen due to overlapping curves, control values are printed beside corresponding curves for aerated cultures. All *Ac* was used after 4 days and so control values are not given from 5th day on. Bracketed number alongside curve refers to number of days' aeration in cases where results from more than one day are presented together.

The alkalinity of all cultures increased steadily from an initial pH value of 6·6 to a relatively constant value of about pH 9·0 by the second day of aeration except in the control where a rapid fall commenced at this time to pH 5·0 which was maintained.

The medium of Expt. 1 is well suited to oxalate formation (80 per cent. yield on acetate supplied or 54 per cent. on carbon basis), but in aerated cultures oxalic acid production was only 50–86 per cent. of that in the control. The smallest yield was provided, as expected, by the culture with most rapid air-flow in which reduced utilization of acetate occurred. Citrate yields were small throughout with this medium which, as already noted, is more conducive to oxalate formation. Nearly as much citric acid was produced in the control as in aerated cultures, except that at the highest rate of aeration (106 ml./min.) no measurable amount of acid was identified.

Pyruvic acid and acetaldehyde were detected on the chromatogram, but only in cultures with slowest air rates (5, 9, and 14 ml. air/min.).



1 day

2 days

3 days

4 days

6 days

7 days

FIG. 2. Metabolism of acetate in Expt. 2 during 7 days' aeration. Aeration rates on a log scale in ml. air/min./culture are shown as abscissae. Ordinates represent pH × 10⁻¹, acetic acid (*Ac*) used, and oxalic (*Ox*) and citric (*C*) acids produced. Other details as in Fig. 1.

Expt. 2. Medium II. This medium which is better adapted to citrate production from acetate was examined as in Expt. 1, except that the range of air flows was increased somewhat, the values being 5 ml. to 500 ml. air min The results (Fig. 2) indicated a similar optimal value for disappearance of acetate as in Expt. 1. Slowing of oxidation was more pronounced at high than at low aeration. As in Expt. 1, acetate consumption was as rapid at the optima rate of aeration as in the culture where free diffusion of air through a cotton-wool plug was allowed.

The rate of oxalate formation like acetate utilization was most rapid with 49 ml. air/min. and in the control. However, even with 5 ml. air/min. the ultimate total oxalate concentration was as great as in the control (or at 49 ml. air/min.), although it required an extra day of growth.

Citrate yields on medium II under optimal aeration (49 ml. air/min.) and in the control were about 1·3 mg. citric acid/ml. and 0·8 mg. citric acid/ml. or 5 per cent. and 3 per cent. on acetate consumed, respectively. These values corresponded to 410 and 250 per cent. of the citrate formed by the control of Expt. 1. The most suitable conditions for citrate synthesis were those under which acetate most rapidly disappeared.

By chromatographic means acetaldehyde was detected but only in the control and in the culture with 49 ml. air/min. No trace of pyruvic acid was obtained in any culture.

B. *The effect of atmospheres containing different concentrations of carbon dioxide*

Expt. 3. Medium II. This series of experiments bore a relationship to Expts. 1 and 2 in that endogenously produced CO_2 in the latter experiments did in fact, by reason of the varying air-flows, set up atmospheres of differing CO_2 content. The present rates of flow were adjusted so that optimal air rate (as found in Expt. 2) was balanced against minimal variable interference from endogenous CO_2 formation. CO_2 was mixed with air and passed at 0 to 12 per cent. concentration over cultures on medium II (Fig. 3). By the third day of aeration the culture receiving 7 per cent. CO_2 accidentally became partially submerged and continued to develop with 70 per cent. of the mycelium just below the surface of the medium. Other cultures gave normal surface growth.

The rate of acetate utilization was slightly greater in cultures with more than 0·6 per cent. CO_2 and was most rapid at the 2 per cent. level. It is possible, however, that cultures receiving the larger amounts of CO_2 would metabolize the acetate more rapidly if the aeration rate were to be increased somewhat.

In the culture with 12 per cent. CO_2 the pH increased gradually from an initial value of 6·8 to a steady level around 8·9, whereas solutions with smaller concentrations of CO_2, after reaching a pH of 8 to 9, later reverted (4 days' aeration) to more acidic conditions (pH 5 to 6).

Inspection of the citrate yields shows that the culture with 2 per cent. CO_2 provided the maximum yield, achieved most rapidly. Cultures with higher or lower gas concentrations provided smaller amounts of citrate and required a longer metabolic period to attain their maxima. However, the amount of citric acid produced in any of the gas-treated cultures was never less than in the control.

Chromatographic examination of the metabolic products indicated the presence of C_4 acids (succinic, fumaric, and malic) from the first day of aeration, in concentrations which often exceeded that of citrate. Furthermore, the C_4 compounds persisted after citrate and acetate had disappeared, thus suggesting that citrate, in part at least, was being oxidized via succinic, fumaric, and malic acids possibly to oxalate. In this respect it is interesting to note than an increase in oxalate formation did occur soon after citrate had reached its maximum and that in some instances the gain and loss of these two acids was roughly related.

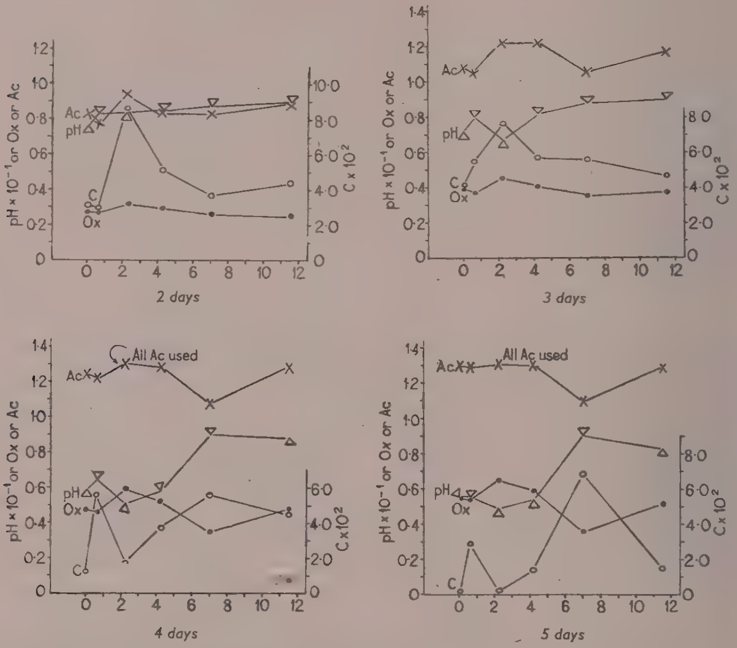


Fig. 3. Metabolism of acetate in Expt. 3 in presence of added CO_2. Percentages CO_2 in entering gas stream are shown as abscissae. Aeration rate to all cultures, 52 ml./min. Ordinates and symbols as with Fig. 1. By the third day of aeration the culture with 7 ml. CO_2 per 100 ml. air had become partly submerged (see text).

DISCUSSION

It is clear that the CO_2 pressure partly regulates the metabolism of *A. niger*. Slow air rates tend to produce the same effects as an increase in concentration of CO_2 and may be presumed to act by the same mechanism.

In Expt. 2 the slower air rate (18 ml./min.) was found to give a similar yield of citrate to that obtained under optimal conditions (49 ml. air/min.), the metabolic period merely being extended. This suggests that a saturation point had been attained for optimal CO_2 concentration. By such regulation of air-rates it was possible to increase citrate formation 64 per cent. over that obtained by free diffusion of air through a cotton-wool plug. Even very slow rates of aeration (5 ml. air/min.), however, provided as much citric acid as was formed under conditions of free diffusion. These observations were confirmed in Expt. 3 in presence of 2 per cent. of CO_2, when an increase of

180 per cent. in citrate production was obtained as compared with the control supplied with CO_2-free air at optimal rate. The effect of aeration and CO_2 on oxalate formation was less pronounced.

The mechanism of the increase in citrate formation is presumably fixation of CO_2, as the progressive increase in acid yield with higher concentrations of gas up to the optimal level (2 per cent. CO_2) would seem to suggest. At greater concentrations of CO_2 the formation of citric acid is reduced, although it is still larger than in the absence of added CO_2. Such a diminution of acid synthesis with large amounts of CO_2 present (or slow air-rates of Expt. 2 at 5 ml./min.) may be attributed to an inhibition of a type similar to that encountered by Foster and Davis (1949) when working with *Rhizopus nigricans*. Isotopic evidence for the role of acetate and CO_2 in the formation of citrate and oxalate by *A. niger* has recently been presented by Foster and Carson (1950), Carson, Mosbach, Phares, Long, and Gwin (1951), Lewis and Weinhouse (1951), and Martin and Wilson (1951).

The presence of pyruvate and its decarboxylation product acetaldehyde when aeration was deficient (Expt. 1) could be ascribed to one or more processes. Chugtai, Pearce, and Walker (1950) have shown that this strain of *A. niger* can produce pyruvate from a glucose-arsenite medium. Alternatively, it is possible in the present work with an acetate substrate that a C_2+C_2 condensation (Thunberg reaction) may have taken place, thus: (*a*) acetate → succinate → fumarate → malate → oxalacetate → pyruvate, or (*b*) malate → lactate → pyruvate, or (*c*) via succinate and citrate to oxalacetate and pyruvate. Finally it would seem permissible also to suggest the possibility that another reaction sequence, involving direct carboxylation of acetate in a manner similar to that originally discovered in certain bacteria, may occur in the mould.

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The Action of Nitrophenols on Respiration and on Glucose Assimilation in Yeast

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SUMMARY

The consumption of 2·25 mg. glucose by a resting suspension of baker's yeast in the presence of finely graded concentrations of 3:5-dinitro-*o*-cresol was followed at pH 2·4 and 5·0 by measuring oxygen uptake and carbon-dioxide output; at pH 8·5 oxygen uptake alone was measured. At each pH certain concentrations of dinitrocresol stimulated respiration and increased the ratio of glucose oxidized to glucose assimilated; at higher concentrations, assimilation was reduced to zero, but respiration became inhibited and the whole of the glucose was then metabolized by aerobic fermentation. Dinitrocresol is believed to be acting in these experiments as an uncoupling agent, lowering the net rate of formation of energy-rich phosphate and so allowing respiration to proceed faster while assimilation, an endergonic process, is inhibited.

The maximum respiratory stimulation obtainable at pH 8·5, 325 per cent. of the control, is greater than can be obtained at pH 2·6 or 5·0, which may be due to the slower respiration rate of controls at pH 8·5. The slow endogenous respiration can also be stimulated more than the exogenous.

With *o*- and *p*-nitrophenols similar relations are obtained, but phenol does not stimulate respiration, although it inhibits assimilation in lower concentrations than respiration.

Under nitrogen, a stimulation of fermentation can be obtained with dinitrocresol at pH 5·0 but not at 2·6. At both pH levels, assimilation is more easily suppressed than fermentation rate.

INTRODUCTION

It was discovered in the 1930's that resting suspensions of fungi and bacteria oxidize only a portion of the organic carbon compound with which they are supplied. A part is oxidized and the remainder is assimilated, being converted to other substances so that there is a gain in dry weight. Thus Pickett and Clifton (1943) found that, under the conditions of their experiments, yeast oxidized about one-third of the glucose that was supplied, half of the remainder being accounted for by the synthesis of an unidentified carbohydrate while a half remained as soluble end-products in the medium. In yeast a comparable process of assimilation occurs under nitrogen, and Fales (1951) and Trevelyan *et al.* (1952) have investigated the properties of the carbohydrates that are formed. It is evident that during assimilation some of the glucose must suffer breakdown by respiration or fermentation to provide the energy which is needed to force the remainder along endergonic pathways such as polysaccharide accumulation.

Many workers have shown that assimilation can be prevented by poisons

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such as 2:4-dinitrophenol and azide, the whole of the substrate being oxidized in the presence of appropriate concentrations. The process of assimilation and the action of inhibitors have been reviewed extensively by Clifton (1946, 1951).

The present investigation concerns the action of nitrophenols, and particularly 3:5-dinitro-*o*-cresol, as inhibitors of the assimilation of glucose by yeast. In the past it has been the practice to test the effect of such inhibitors by applying them at, say, three or four concentrations only, and the greatest stress has been placed on concentrations which reduce the rate of respiration. It is, however, well known that certain concentrations of nitrophenols stimulate the rate of respiration to a marked degree and there has been considerable speculation as to the mechanism of this stimulation. A recent view postulates control of the rate of respiration by the phosphorylation cycle (Johnson, 1941). Under normal conditions the rate is limited by the availability of acceptors for the large quantity of energy-rich phosphate formed during oxidation. Loomis and Lipmann (1948) have shown that dinitrophenol can prevent the formation of energy-rich phosphate during oxidation *in vitro*. If the same is true *in vivo* the stimulation of respiration by dinitrophenols could be attributed to the reduced efficiency of phosphorylation. A further consequence of reduced phosphorylation would be an inhibition of energy-requiring processes, of which assimilation is one. The purpose of the present paper is to report experiments on the effects of dinitrophenols on respiration, fermentation, and assimilation in yeast and to discuss the significance of the phosphorylation cycle in these effects.

EXPERIMENTAL METHODS

Initial experiments were carried out with ordinary commercial baker's yeast and were later confirmed and extended using a pure yeast isolated from a baker's yeast. The results obtained with these two yeasts showed only minor differences, and except where otherwise stated, the experiments reported here were all performed with the pure yeast.

The assimilation of small amounts of glucose was studied by measuring oxygen consumption at 25° C. with a Warburg manometric apparatus. The flasks each contained 2 ml. 1 per cent. yeast in M/15 phosphate buffer and the desired concentration of nitrophenol in 0·5-ml. buffer. In the experiments at pH 2·4 and 5·0 carbon dioxide production was measured by parallel flasks from which potash was omitted. When the flasks had reached equilibrium in the water-bath, 2·25 mg. glucose in 0·5 ml. water was added from the side-arm, and measurements were then taken at frequent intervals for several hours until steady rates of oxygen uptake and carbon dioxide output were reached.

In the experiments designed to study assimilation and the rate of fermentation under nitrogen a similar procedure was followed, the flasks being gassed with oxygen-free nitrogen, allowed to equilibrate, and the glucose then tipped in from the side-arm.

EXPERIMENTAL RESULTS

The oxidative assimilation of glucose by yeast was investigated by measurements of oxygen uptake following the addition of 2·25 mg. glucose. Complete oxidation of this quantity of glucose according to the equation

$$C_6H_{12}O_6+6O_2 = 6H_2O+6CO_2$$

would result in an uptake of 1835 μl. oxygen at 25° C.

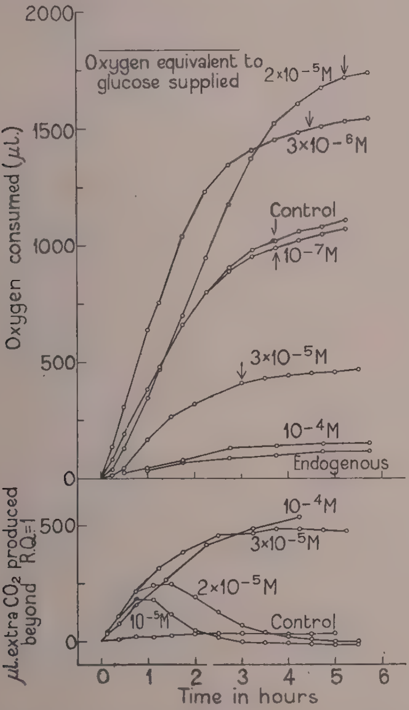

FIG. 1. The effect of various concentrations of 3:5-dinitro-*o*-cresol on oxygen uptake (above) and carbon dioxide production beyond that required for R.Q. of unity (below) following the addition of 2·25 mg. glucose to a yeast suspension at pH 2·4.

Four experiments were carried out at pH 2·4, each with a control and a range of concentrations of 3:5-dinitro-*o*-cresol, and a selection of the results is shown in Fig. 1 as progress curves of oxygen uptake against time. The curves for the control and for low concentrations of inhibitor are of the type described by earlier workers (Clifton, 1946), showing an initial phase of rapid oxygen uptake, and then a break in the curve followed by a much slower respiration rate. It has been shown (Clifton, loc. cit.) that the position of this break corresponds to exhaustion of the glucose from the external medium.

In order to obtain a quantitative measure of assimilation the time when the change in rate occurs was defined by the point at which the rate of oxygen uptake first fell to less than 65 ml. per hour per 20 mg. wet wt. aliquot of yeast. The points at which the curves in Fig. 1 reached this rate are indicated by arrows. No allowance was made for the endogenous oxygen consumption; it was comparatively small in amount and is probably suppressed in the presence of exogenous substrate (Syrett, 1951). It will be seen from the graph that the oxygen consumption of the control is just over a half of that required for complete oxidation and that the presence of 10^{-7} M. dinitrocresol has little effect on oxygen consumption. The respiratory quotient was approximately unity throughout the experiment in the controls (Fig. 1) and with low concentrations of inhibitor.

With higher concentrations such as 10^{-5} M. and 2×10^{-5} M. a larger proportion of the glucose was respired and over an initial period the R.Q. was as high as 1·5–2·0. Krahl and Clowes (1935) have demonstrated the accumulation of alcohol in the presence of dinitrocresol (at concentrations above 5×10^{-6} M. at pH 4·45) so that the high R.Q. values may be ascribed to the appearance of aerobic fermentation. The period of high R.Q. lasts only about 1½ hours and is followed by low R.Q. values (0·7–0·8) and eventually after 4–5 hours by a R.Q. of unity. It appears that respiration is at first accompanied by aerobic fermentation with accumulation of alcohol, but after about an hour, as the hexose concentration begins to fall, the alcohol is oxidized; a comparable finding is reported by Swanson and Clifton (1948), who found that the R.Q. of growing yeast cultures is at first high as a result of fermentative assimilation but the period of high R.Q. only lasts while there is an appreciable amount of reducing sugar present, being followed by oxidative assimilation of the accumulated intermediate products including alcohol.

With the highest concentrations of inhibitor illustrated in Fig. 1, 3×10^{-5} M. and 10^{-4} M., the rate of oxygen uptake was relatively low and diminished gradually during the course of the experiment. The R.Q. was high, giving values between 2 and 6 over a long initial period, after which it gradually declined to unity. These results suggest that although a considerable quantity of alcohol was produced in the initial period, prolonged treatment with high concentrations of dinitrocresol may impair the ability of the cells to oxidize the alcohol at a later stage when the supply of hexose becomes exhausted, either reducing the rate of oxidation or preventing it altogether.

It is seen from Fig. 1 that concentrations up to 2×10^{-5} M. cause a progressive increase in the percentage of glucose that is oxidized. Although there is an initial period of aerobic fermentation at 10^{-5} M. and 2×10^{-5} M., this is compensated by the subsequent oxidation of the alcohol formed, and the overall R.Q. is unity. As much as 93 per cent. of the glucose is oxidized with 2×10^{-5} M. With the highest concentrations only a small proportion of the glucose is oxidized, but a relatively large amount is fermented. Thus at 3×10^{-5} M., 21 per cent. of the sugar is oxidized and 74 per cent. fermented.

Confirmation of the general thesis that the low rates of oxygen uptake and carbon-dioxide evolution indicate exhaustion of the glucose was obtained in an experiment with baker's yeast at pH 3·0 by measurements of the reducing sugar content of the medium after the period of rapid oxygen uptake. The quantity of sugar found in the control media and also in the presence of 10^{-5} M. and 5×10^{-5} M. inhibitor was less than 5 per cent. of that originally present in the medium.

The curves shown in Fig. 1 were used to measure the effect of dinitrocresol on the respiration rate of yeast as well as its effect on oxidative assimilation. The rate of oxygen uptake was relatively low for the first 10 minutes after adding the glucose, but there followed a period when the uptake remained linear until it finally began to decline. Respiration rate was measured by the oxygen uptake in the first 30 minutes of the linear period. From the carbon dioxide production over this period in excess of that required for a R.Q. equal to that of the controls, it was possible to obtain some indication of the rate of aerobic fermentation; but as the rate was not always constant over the whole 30-minute period, this measure is only approximate.

The results of the whole series of experiments on the inhibition of glucose assimilation at pH 2·4 are set out in Fig. 2. The upper curve shows the effect of increasing concentrations on the respiration rate and is characterized by a well-marked stimulation, the maximum rate being 1·73 times that of the control. The action of dinitrocresol as an inhibitor of oxidative assimilation has been expressed by dividing the percentage of glucose assimilated in the presence of the concentration in question by the percentage assimilation in the control. The curve only shows concentrations up to 2×10^{-5} M., for which the overall process of assimilation is purely oxidative with R.Q. of unity. It can be seen that as the concentration is raised there is a progressive inhibition of assimilation; and that the inhibition is brought about by the same concentrations as are needed to stimulate respiration—from about 10^{-7} M. onwards. The proportion of glucose assimilated is reduced to one-half by a concentration of inhibitor (2×10^{-6} M.) which is a little less than that needed for maximal stimulation of respiration (5×10^{-6} M.). At concentrations above this, respiration is inhibited and aerobic fermentation commences, but is in turn suppressed by yet higher concentrations.

To determine whether these relationships could be regarded as typical, the investigation was widened by studying, first, the assimilation of glucose at other pH levels, then by using other nitrophenols, and finally by experiments under nitrogen.

The results obtained at pH 5·0 (Fig. 3) are on the whole very similar to those from the experiments at pH 2·4, although higher concentrations of dinitrocresol are required. The maximal stimulation of respiration is given at 2×10^{-5} M. and amounts to a rate 1·95 times that of the control. Oxidative assimilation is halved by a slightly lower concentration, and here, again, aerobic fermentation appears at concentrations of inhibitor which reduce the rate of respiration.

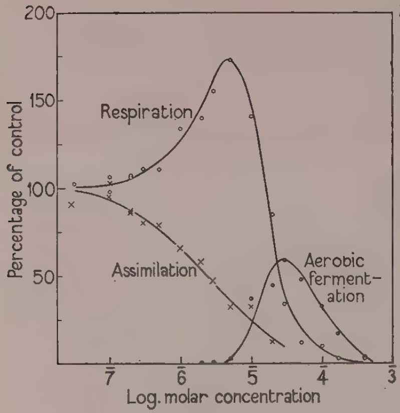

FIG. 2.

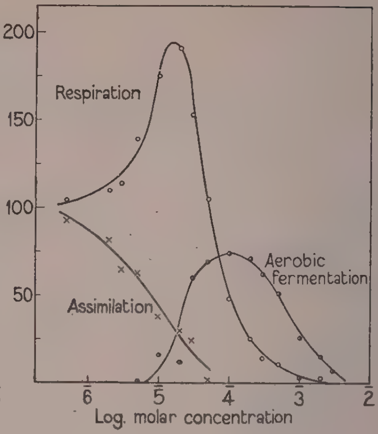

FIG. 3.

FIG. 2. Effect of 3 : 5-dinitro-*o*-cresol on respiration rate and the proportion of glucose assimilated by yeast at pH 2·4. The rate of aerobic fermentation is shown as a percentage of the carbon dioxide production of the control. Mean R.Q. of controls = 1·07.

FIG. 3. As Fig. 2. Results of an experiment at pH 5·0. Mean R.Q. of controls = 1·00.

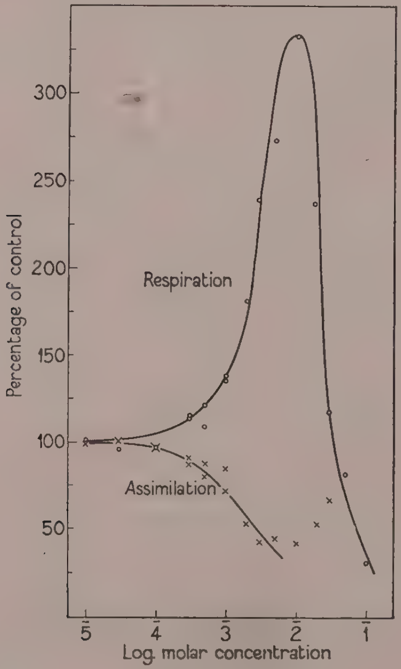

FIG. 4. Effect of 3 : 5-dinitro-*o*-cresol on respiration rate and the assimilation of glucose by yeast at pH 8·5.

Another series of experiments was run at pH 8·5 (Fig. 4), using concentrations of dinitrocresol some 1,000–2,000 times greater than were required at pH 2·4. The degree of stimulation is much enhanced, reaching a level 3·35 times greater than the control at 10^{-2} M., while oxidative assimilation is halved by 3×10^{-3} M. The curve for percentage inhibition of oxidative assimilation does not show a steady and progressive decline with increasing concentrations of dinitrocresol, probably because of the appearance of aerobic fermentation at higher concentrations, only a part of the glucose being respired and the rest consumed by fermentation together with a small amount of assimilation. No measurements of the course of carbon dioxide production were made at pH 8·5, so that this suggestion remains unconfirmed. It is also possible that the highest concentrations of inhibitor cause a gradual paralysis of the enzymes responsible for oxidation so that assimilation comes to a standstill before it is complete.

Glucose assimilation is shown in the preceding graphs as a percentage of the amount of assimilation in the control. This amount varies with pH (Table I), being about 65 per cent. over the median pH range, but only 42 per cent. at the lowest pH.

TABLE I

Effect of pH on the percentage of the glucose supplied to yeast that is assimilated

pH	2·4	5·0	5·7	8·5
No. of observations . . .	5	4	1	8
Mean % assimilation . . .	42·1	64·0	62·9	67·1

The effects of phenol and *o*- and *p*-nitrophenols on respiration and assimilation were examined at pH 8·5, the results being shown in Fig. 5. The curves for *p*-nitrophenol are similar in many respects to those for dinitrocresol, showing a stimulation of respiration associated with inhibition of oxidative assimilation and an inhibition of respiration at higher concentrations. The effects of *o*-nitrophenol on metabolism are probably of the same nature, but only a small part of the curves can be investigated at pH 8·5 owing to the relatively low solubility of *o*-nitrophenol—just above 10^{-2} M. under the experimental conditions. The curve for phenol, on the other hand, shows no stimulation; the respiration remains at a steady rate until a concentration of 3×10^{-2} M. is reached, when it falls sharply. Assimilation is cut down by concentrations above 5×10^{-2} M. At the highest concentrations of phenol (and also with high concentrations of *p*-nitrophenol) there is a rise in the curve for percentage assimilation, which again may be ascribed to the appearance of aerobic fermentation or perhaps to a gradual injury of the oxidizing enzymes.

The effects of 3:5-dinitro-*o*-cresol on fermentation rate and on the fermentative assimilation of glucose were next investigated. The Warburg flasks were flushed with nitrogen, equilibrated, and glucose was then added from the side-arm in the same quantity as was used in the respiration experiments, namely 2·25 mg. per flask. Complete fermentation according to the equation

$$C_6H_{12}O_6 = 2C_2H_5OH+2CO_2$$

would yield 612 μl. CO_2 at 25° C. A series of curves comparable to those of Fig. 1 were obtained and analysed in the same way. The quantities of glucose assimilated were small, the percentage assimilation in the controls being 23 per cent. at pH 2·6, 26 per cent. at pH 3·0, and 30 per cent. at pH 5·0 (each figure the mean of two determinations). The rate of carbon dioxide production was at first high, but it began to decrease so soon after the addition of glucose that it was necessary to make the estimate of fermentation rate from the carbon dioxide production over a period of only 20 minutes.

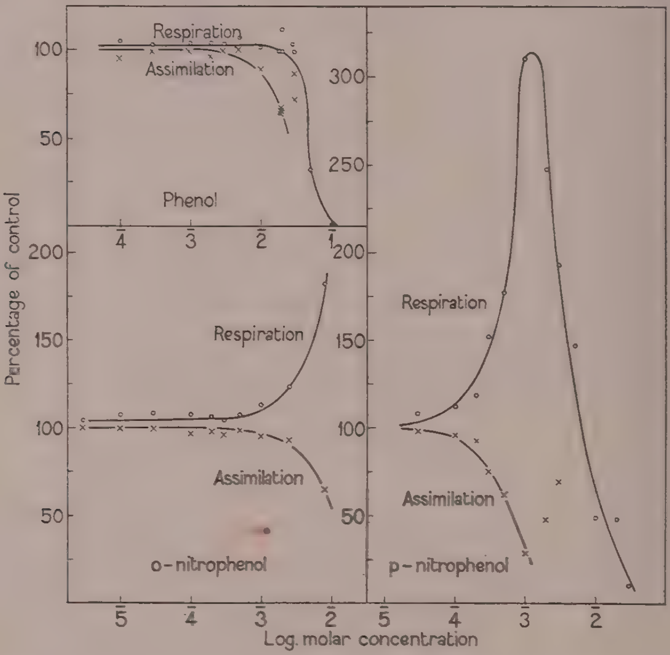

FIG. 5. Effect of phenol and *o*- and *p*-nitrophenols on respiration rate and the assimilation of glucose by yeast at pH 8·5.

It is clear from Fig. 6 that at pH 2·6 there was no stimulation of fermentation, the rate steadily decreasing in concentrations above about 3×10^{-6} M. Assimilation was inhibited at concentrations above 10^{-6} M. Comparable results were recorded in two experiments at pH 3·0, one with the pure yeast and one with bakers' yeast. In the experiment at pH 5·0 a stimulation of the rate of fermentation was observed, the maximum rate being 118 per cent. of that of the control. Stimulation of fermentation and inhibition of the assimilation of glucose both became apparent at concentrations above about 3×10^{-6} M. In both experiments there is an apparent rise in the percentage of glucose assimilated at high concentrations. This must be ascribed to progressive injury to the enzymes concerned in fermentation leading to premature arrest of glucose consumption. At the highest dinitrocresol concentrations the rate of

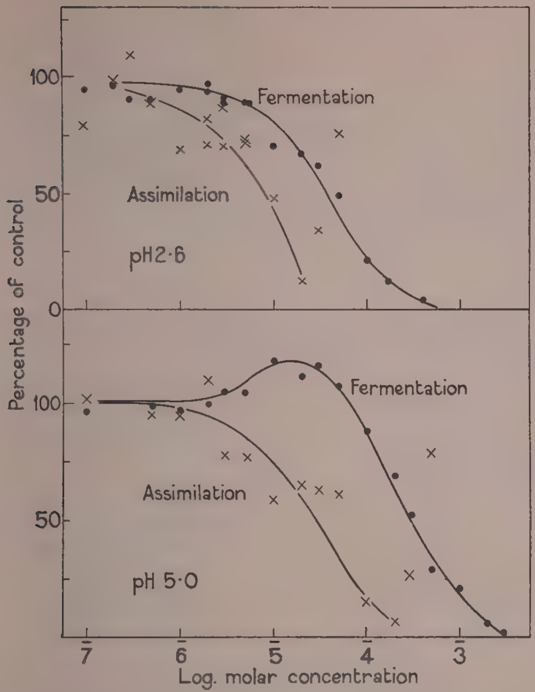

FIG. 6. Effect of 3:5-dinitro-*o*-cresol on fermentation rate and the proportion of glucose assimilated by yeast under nitrogen at pH 2·6 (above) and pH 5·0 (below).

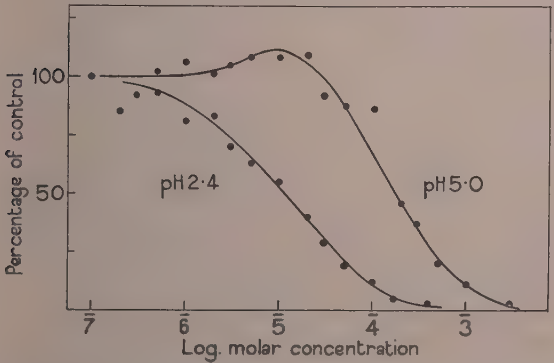

FIG. 7. Effect of 3:5-dinitro-*o*-cresol on the rate of fermentation of yeast under nitrogen at pH 2·4 and 5·0.

fermentation is very low, falling below the standard figure of 65 ml./hour per 20 mg. yeast from the start of the experiment so that no glucose at all is assimilated.

That the rate of fermentation is stimulated by dinitrocresol at pH 5·0 but not at the lower pH was confirmed in two experiments in which the glucose concentration was increased to 0·2 M. and carbon dioxide production was measured over a 30-minute period. The results show a stimulation of 111 per cent. at pH 5·0, but none at pH 2·4 (Fig. 7). Krahl and Clowes (1935) found that they were able to convert a yeast which showed no stimulation at pH 4·45 to one that gave a well-marked stimulation by soaking it overnight in glucose buffer. This result was confirmed in one experiment in which a stimulation of as much as 130 per cent. was obtained after 15 hours' soaking in glucose at pH 5·0.

DISCUSSION

The experiments described above deal with the effects of nitrophenols on the oxidative and fermentative assimilation of glucose by yeast and corresponding measurements were made at the same time on respiration and fermentation.

Dinitrocresol experiments in air

Respiration. The series of experiments at pH 2·4, 5·0, and 8·5 all show a well-marked stimulation of respiration followed at higher concentrations by an inhibition, the concentrations required for stimulation increasing as the pH is raised. The extent of stimulation is also greater in alkaline solution, the maximum respiration rate being 335 per cent. of the control at pH 8·5, but only 195 per cent. at pH 5·0 and 173 per cent. at pH 2·4. There is a corresponding difference in the range of concentration over which stimulation is shown. At pH 8·5 the respiration rate exceeds 110 per cent. of the control between concentrations of 2×10^{-4} M. and 3×10^{-2} M., i.e. over a range of 150 times; the range at pH 5·0 is 30 and at pH 2·4 it is 40. These figures implement the obvious suggestion that the curve showing respiratory stimulation is the resultant of two trends, one for stimulation and another, which only becomes important at higher concentrations, for inhibition. It might be that despite the uniformity of the effect of pH on weak acids (Simon and Beevers, 1952), a change of pH has slightly different effects on the concentrations of dinitrocresol which elicit the two trends, the concentrations needed to inhibit respiration being increased by a larger amount for a given rise in pH than those needed for stimulation. This could account for the enhanced degree of stimulation at pH 8·5 and for the wider concentration range over which stimulation is apparent. Another possibility was suggested by the experiment set out in the following paragraph.

It was found that the maximum respiration rate attainable with dinitrocresol could be altered by a factor other than pH. In one experiment the effect of increasing concentrations on the endogenous respiration of baker's yeast was measured first; glucose was then added from the side-arm and the measurements repeated on the exogenous respiration. The results are presented in Fig. 8A. The endogenous respiration reaches its peak at a concentration of 10^{-4} M., but on the addition of glucose the peak is found at a lower concentration (2×10^{-5} M.). At a concentration such as 10^{-4} M., dinitrocresol stimulates endogenous respiration but inhibits exogenous respiration. A simple interpretation of this result comes from a consideration of the actual rates of oxygen uptake (Fig. 8 B). Although those portions of the curves which represent stimulation of respiration occupy widely different parts of the graph, exogenous respiration being some 9 times faster than endogenous, the curves representing inhibition of respiration are closely aligned. The relationship between concentration and inhibition of respiration is of the usual sigmoid form so that if the control respiration is slow, like the endogenous respiration, a high concentration of dinitrocresol must be reached before the inhibitory effect becomes apparent. The maximum respiration rate therefore occurs at a relatively high concentration, and since percentage stimulation rises with increasing concentration, it is greater for a slow respiration than for a fast one.

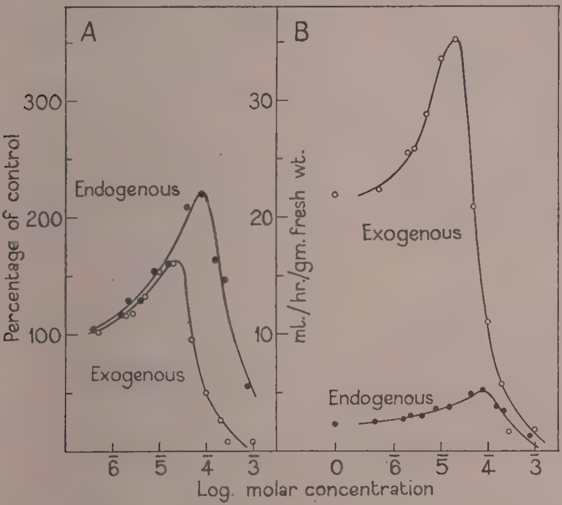

FIG. 8. Effect of 3:5-dinitro-*o*-cresol at pH 5·0 on the rate of oxygen uptake of baker's yeast in the absence of external glucose (closed circles) and in the presence of 0·2 M. glucose (open circles). A. Expressed as percentage of the control rates. B. The absolute rates.

The relatively high degree of stimulation obtained at pH 8·5 may be related to respiration rate in a similar way since the rate of respiration at pH 8·5 was 4·8 μl. min. per 20 mg. aliquot of yeast as compared with rates of 6·6 and 7·4 at pH levels of 5·0 and 2·4 respectively.

Aerobic fermentation. Measurements of R.Q. in the experiments at pH 2·6 and 5·0 showed that aerobic fermentation appeared at concentrations of dinitrocresol which inhibit respiration. Although the rates of aerobic fermentation are indicated in Figs. 2 and 3, it is again stressed that these values are only approximate owing to the rather rapid variations in R.Q. found at some concentrations (cf. Fig. 1). To obtain a more reliable measure of the release of aerobic fermentation a comparable series of experiments was performed in the presence of an excess of glucose. The results of these experiments are reported in the following paper.

Oxidative assimilation. The quantity of glucose supplied to the yeast was the same in all experiments, but the manner in which it was utilized by the yeast was influenced by two factors—pH and the presence of nitrophenols.

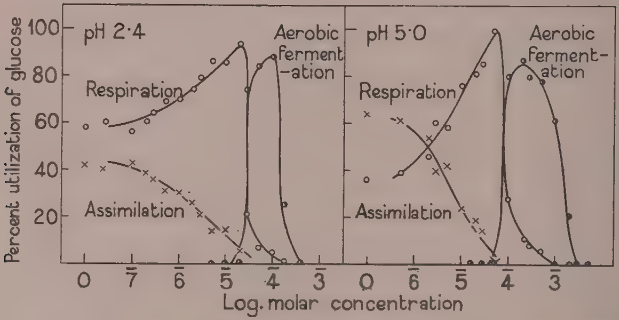

FIG. 9. Effect of 3:5-dinitro-*o*-cresol on the metabolism of a small quantity of glucose (2·25 mg.) supplied to a yeast suspension at pH 2·4 and at 5·0. There are three processes by which glucose may be consumed—assimilation, respiration, and aerobic fermentation.

In the controls the whole of the glucose is accounted for by the two processes of respiration and assimilation and it is here that the operation of the pH factor is most evident. In the most acid solutions more glucose was respired and less assimilated than at other pH levels. It may be noted that the reverse relationship was observed by Taylor (1950) for the assimilation of glucose by *Scenedesmus quadricauda*.

With increasing concentrations of dinitrocresol less and less glucose is assimilated until a point is reached at which nearly all of the glucose is respired (Fig. 9). A slight increase in concentration beyond this point is sufficient to reduce the rate of respiration, to inhibit respiratory consumption of glucose, and to cause the appearance of aerobic fermentation which then accounts for the major part of the glucose that is consumed. With yet higher concentrations there is a reduction in the rate of aerobic fermentation and in the amount of glucose consumed in the fermentation process.

As was pointed out above, the proportion of glucose assimilated has been calculated in this investigation as 100 minus the percentage of glucose respired, a treatment which obviously applies only to that concentration range in which assimilation and respiration are the sole pathways of metabolism for the glucose. At the lowest concentrations at which there is aerobic fermentation, percentage assimilation could still be calculated as 100 minus per cent. respired minus per cent. fermented. However, caution is necessary in using this formula since it is clear that concentrations of dinitrocresol which inhibit both respiration and fermentation must also inhibit assimilation, so that there would be included in the figure for percentage assimilation the glucose that remains unaltered in the medium: under these conditions the equation should read

per cent. assimilated = 100—per cent. respired—per cent. fermented—per cent. unaltered.

Thus at pH 2·4, with 4×10^{-4} M. dinitrocresol when respiration and fermentation are both strongly inhibited, the percentage assimilation is not 100, but zero. To avoid this possible source of error in the experiments at pH 2·4 and 5·0, percentage assimilation was calculated only for concentrations which had an overall R.Q. of 1 and in which glucose was consumed either by respiration or assimilation. In the several experiments at pH 8·5, however, where no measurements of R.Q. were made, no account could be taken of aerobic fermentation (or of progressive injury to the enzymes of catabolism), so that there is an apparent increase in the percentage assimilated at the highest concentrations. Similar considerations apply to the experiments on assimilation under nitrogen, where no account could be taken of progressive injury to the enzymes of fermentation.

It may be noted here that the calculations of respiration and fermentation, and hence of assimilation as well, all depend on the assumption that the usual equations for respiration and alcoholic fermentation are a true representation of the rates of oxygen uptake and carbon dioxide output at the various pH levels used and in the presence of nitrophenols.

There has been some discussion in the literature as to whether dinitrophenols inhibit the assimilation of glucose supplied exogenously or whether they stimulate the utilization of the material that has just been assimilated. Evidence that the materials formed during a period of assimilation can be utilized comes from experiments in which the addition of a dinitrophenol after a period of glucose assimilation is found to cause an extra uptake of oxygen so that the total oxygen consumption is the same as it would have been if the dinitrophenol had been added at the start of the experiment (Burris and Wilson, 1942). It is also true that dinitrophenols can stimulate the respiration of the endogenous reserves of ordinary parlance, those which were laid down in a much earlier period of assimilation and of which some may have since been consumed in endogenous respiration or been converted into substances such as structural polysaccharides which are immune to the action of dinitrophenols. Respiration presumably continues until the reserves have fallen to a lower level at which they remain stable. On the other hand, when dinitrophenol is applied to a well-starved yeast that is being fed with glucose, it limits the quantity of reserve that can be built up, causing the remainder of the glucose to be oxidized. It is not unreasonable to suppose that, associated with each dinitrophenol concentration, there is a characteristic level at which the carbohydrate reserves can be maintained. When dinitrophenol is added to yeast the carbohydrate reserves approach this level either by consumption of the excess carbohydrate or with a starved yeast by inhibition of more than the appropriate amount of assimilation. The two processes can proceed at once if dinitrophenol is applied to a micro-organism which already contains large endogenous reserves and is at the same time being fed with glucose (Stout and Koffler, 1951).

Relation between the stimulation of respiration and oxidative assimilation. The experiments reported above make it clear that respiratory stimulation and oxidative assimilation are closely connected. In each experiment the two processes commence at about the same dinitrocresol concentration, indicating that they are due to the same type of toxic action. The opposite state of affairs is shown by the relation between the stimulation of respiration and the inhibitory process which are believed to be due to different types of toxic action because they affect respiration rate over different concentration ranges. For this reason, again, it is held that the inhibition of assimilation and the inhibition of respiration are distinct processes: at each pH assimilation is 50 per cent. inhibited by dinitrocresol concentrations less than are required for maximal stimulation and hence too low for much respiratory inhibition to be in evidence.

There can be little doubt that stimulation of respiration and inhibition of assimilation are both due to the action of dinitrocresol on oxidative phosphorylation. Since the original publication of Loomis and Lipmann in 1948 several workers have shown that dinitrophenols interfere with the normal working of cell-free systems capable of carrying out oxidative phosphorylation (e.g. Cross *et al.*, 1949; Judah, 1951). In the presence of dinitrophenols there is a fall in the ratio of moles of phosphate esterified to moles of oxygen taken up, indicating that the production of energy-rich phosphorus compounds has been inhibited. This has two consequences.

First, endergonic processes which are driven by energy-rich phosphate will be inhibited. Assimilation is such a process since it involves the conversion of glucose into polysaccharides and other metabolites along pathways in which there is little or no decrease of free energy.

The stimulation of respiration by dinitrophenols may also be ascribed to their effect on phosphorylation, since it is now generally agreed that provided there is an ample supply of substrate the rate of respiration is governed by the supply of acceptors for energy-rich phosphate. The rate of respiration increases when the cell is doing work since utilization of energy-rich phosphate releases fresh supplies of phosphate acceptors and allows further phosphorylation to occur. The rates of both glycolysis (Dixon, 1949) and oxidation (Lardy and Wellman, 1952) are controlled in this way. Hence if less energy-rich phosphate is produced during respiration in the presence of a dinitrophenol, the respiration rate will rise by a corresponding amount. The present experimental results make it clear that increasing concentrations of dinitrocresol cause progressively more and more severe inhibition of the production of energy-rich phosphate with the result that percentage stimulation of respiration and percentage inhibition of assimilation increase *pari passu*. This relationship only holds up to concentrations at which the respiration rate becomes inhibited; consideration of the effects of higher concentrations involves a discussion of aerobic fermentation and is deferred to the next paper.

Comparable observations have been made by other workers, the most extensive being those made by Krahl and Clowes and their colleagues (see Krahl, 1950) with fertilized Echinoderm eggs. As the concentration of dinitrophenol was increased there were parallel increases in the rate of respiration and in the degree of inhibition of cell division. These experiments and others of a similar nature are discussed more fully elsewhere (Simon, 1953).

Relative activity of different compounds. The experimental results obtained with *p*-nitrophenol are closely similar to those for dinitrocresol at pH 8·5. There is little doubt that *o*-nitrophenol also inhibits oxidative phosphorylation though the concentrations that have to be applied in the external medium approach the limit of solubility. The results obtained with phenol, on the other hand, provide no clear evidence of any effect on phosphorylation.

Several investigators have tested these substances on particulate preparations capable of carrying out oxidative phosphorylation. Clowes *et al.* (1950) found that *p*-nitrophenol, which stimulates the respiration of Echinoderm eggs while preventing cell division, was also active towards cell-free preparations; *o*-nitrophenol, on the other hand, was inactive both *in vivo* and *in vitro* at 10^{-3} M. Cross *et al.* (1949) state that *o*-, *m*-, and *p*-nitrophenols at $2{\cdot}5 \times 10^{-4}$ M., have no effect on phosphorylation by cyclophorase preparations.

Dinitrocresol experiments under nitrogen. The experiments on fermentative assimilation at pH 2·6 provide no evidence that dinitrocresol is interfering with phosphorylation. The fact that assimilation is somewhat more sensitive to dinitrocresol than fermentation is most simply interpreted on the following lines. The several endergonic processes within the cell compete with one another for the energy supply available (Sussman and Spiegelman, 1950). As the rate of fermentation is reduced by dinitrocresol so the energy-supply becomes limited and some processes, including assimilation, are inhibited to a relatively large degree.

At pH 5·0, not only was assimilation much more sensitive to dinitrocresol than fermentation, but there was a definite stimulation of fermentation suggesting an inhibition of the production of energy-rich phosphate during glycolysis under nitrogen. The results of Reiner and Spiegelman (1947) and Rothstein and Berke (1952) showing that dinitrophenol can stimulate the fermentation of endogenous reserves under nitrogen also suggest, by analogy with the aerobic experiments, that dinitrophenols prevent glycolytic phosphorylation. This possibility is considered in more detail in a general discussion of the toxic actions of dinitrophenols (Simon, 1953).

Preliminary experiments were carried out at Oxford while I was a member of the Agricultural Research Council Unit of Experimental Agronomy, and the main part of the work was completed in London during tenure of a Senior Studentship awarded by the Royal Commission for the Exhibition of 1851.

I wish to thank Professor G. E. Blackman and Professor T. A. Bennet-Clark for encouragement and stimulating discussions.

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Dinitrocresol, Cyanide, and the Pasteur Effect in Yeast

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SUMMARY

At pH 5·0 the respiration of yeast is stimulated by low concentrations of 3:5-dinitro-*o*-cresol, reaching a peak level of 170 per cent. at 10^{-5} M. Concentrations above this inhibit oxygen uptake and cause aerobic fermentation to appear, which in turn reaches a peak value and is then inhibited. The rate of carbohydrate breakdown, or glycolysis, calculated from the rates of respiration and aerobic fermentation, increases steadily up to 3×10^{-5} M., at which concentration it is 5 times faster than the control: higher concentrations depress the rate of glycolysis. The rate of fermentation under nitrogen is about twice that of respiration, and it is inhibited over the same concentration range as aerobic fermentation.

It was found earlier that oxidative assimilation of glucose by yeast is progressively inhibited by increasing concentrations of dinitrocresol, and it is now shown that this parallels the increase in the rate of aerobic glycolysis. It is argued that dinitrocresol is here acting as an uncoupling agent and that both oxidative assimilation and the rate of glycolysis are controlled by the level of energy-rich phosphate.

With cyanide there is no stimulation of oxygen uptake, aerobic fermentation only appears when respiration becomes inhibited, and after an initial slight decrease the rate of glycolysis rises to 575 per cent. of the control value at 5×10^{-4} M. It is suggested that the rate of glycolysis only increases when respiration has been inhibited sufficiently to reduce the rate of formation of energy-rich phosphate.

INTRODUCTION

In his recent review of the Pasteur Effect in plants Turner (1951) distinguishes three principal types of mechanism which could account for the fact that the rate of destruction of glucose by yeast is faster in nitrogen than in air. According to one mechanism, that of oxidative anabolism, some of the products of glycolysis are reconverted to carbohydrate. It has recently become clear that this process is not operative in yeast since Gottschalk (see Turner, loc cit.) has shown that the synthesis of polysaccharide, and indeed of other substances also, is no greater in air or oxygen than in nitrogen. Where the possibility of oxidative anabolism exists, it is difficult to obtain an unequivocal measure of the rate of glycolysis in air because it could be claimed that the rate of oxygen uptake only measures the difference between the rates of glycolysis and oxidative anabolism. However, there is now no difficulty in calculating the

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rates of glycolysis in air and in nitrogen for yeast, from the rates of oxygen uptake and carbon dioxide output. The present investigation concerns the next problem, that of determining how the rate of glycolysis is controlled. Two principal hypotheses are to be considered, that which sees in diphosphopyridine nucleotide (DPN, coenzyme I) the factor controlling the rate of carbohydrate loss (Gottschalk, 1941), and that which lays stress on the phosphate cycle (Johnson, 1941).

The experiments described in this paper were all performed with baker's yeast and developed from an investigation of the effect of 3:5-dinitro-*o*-cresol on the assimilation of glucose by yeast (Simon, 1935*a*). In the presence of concentrations high enough to give a partial inhibition of respiration, an appearance of aerobic fermentation was noticed. Only a small amount of glucose was supplied to the yeast in these experiments, and the fermentation ceased as the glucose became exhausted. It was decided to repeat the experiments, using a wide range of dinitro-cresol concentrations and an excess of glucose. A comparable series of experiments was performed with cyanide, and the results are discussed in the light of our knowledge of the modes of action of these two inhibitors.

EXPERIMENTAL METHODS

Conventional Warburg methods were used to measure the oxygen uptake and carbon dioxide output of a pure yeast isolated from baker's yeast. All the experiments were run at pH 5·0 and at 25° C. In the respiration experiments with 3:5-dinitro-*o*-cresol each flask contained 2 ml. 1 per cent. yeast in M/15 phosphate buffer with M/6 glucose and the desired concentration of inhibitor was added in 0·5 ml. buffer. Fifteen minutes was allowed for equilibration and oxygen uptake was then measured in the following 30 minutes. Carbon dioxide output was measured by parallel flasks from which potash was omitted. In the experiments with cyanide the concentration of yeast was halved in order to reduce the rate of evolution of carbon dioxide and Kreb's (1935) mixtures were used in the centre well. At the two highest concentrations (3×10^{-3} and 10^{-2} M.) a different method was used, 5 per cent. potash being placed in the centre well after 25 minutes' equilibrium on the water-bath. With this procedure errors could arise as a result of distillation of cyanide from the medium into the centre well which would lower the effective cyanide concentration and so give an overestimate of oxygen uptake; however, these high cyanide concentrations gave a complete inhibition of respiration, no oxygen uptake being observed.

In the experiments under nitrogen the flasks were set up as above, with the exception that the glucose was held in the side-arm; after gassing with oxygen-free nitrogen and allowing a period for equilibration, the glucose was tipped in and the period of fermentation allowed to begin. Carbon dioxide output was measured in the next half-hour. With this procedure the period of fermentation was not preceded by a recent experience of exogenous respiration.

EXPERIMENTAL RESULTS

The effect of increasing concentrations of 3 : 5-dinitro-*o*-cresol on the oxygen uptake and carbon dioxide output of yeast was measured first. Carbon dioxide output beyond that required for a respiratory quotient equal to that of the controls (R.Q. = 1·05) is ascribed to aerobic fermentation and has been plotted in Fig. 1 A as apercentage of the respiratory carbon dioxide output of

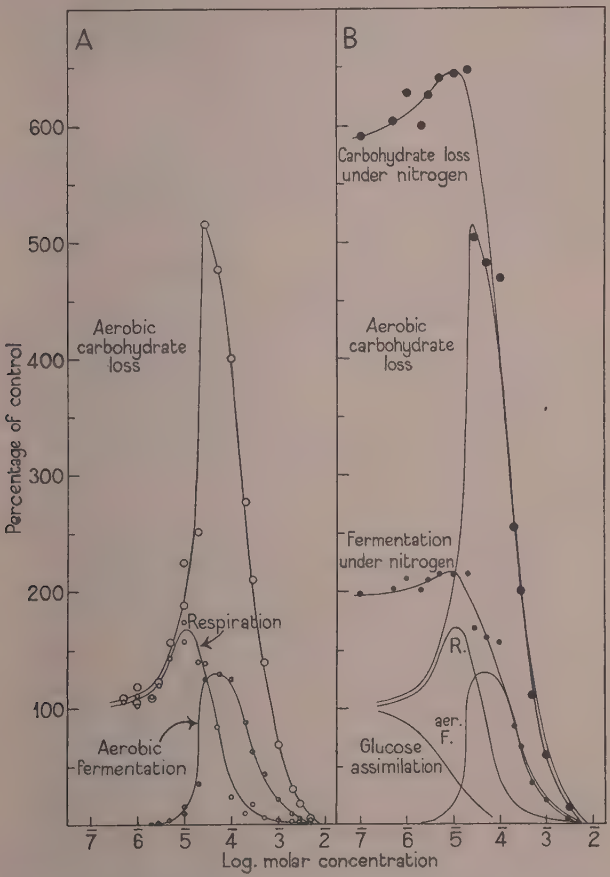

FIG. 1. Effects of 3 : 5-dinitro-*o*-cresol at pH 5·0 on yeast metabolism. A. In air. Respiration, measured by O_2 uptake (mean rate in controls = 29·6 ml./hr.g. fresh wt.); aerobic fermentation, measured by CO_2 production and expressed as a percentage of respiratory CO_2 production in the controls; and rate of carbohydrate loss calculated from the rates of respiration and aerobic fermentation and expressed as a percentage of the controls. B. Respiration, aerobic fermentation, and rate of carbohydrate loss as in A. Rate of fermentation under nitrogen measured by CO_2 output and expressed as a percentage of the rate of CO_2 output by controls in air; rate of carbohydrate loss under nitrogen expressed as a percentage of the corresponding rate in air. Oxidative assimilation of glucose by yeast (from Simon, 1953, Fig. 3).

the control. It will be seen that respiration is stimulated at the lower concentrations but progressively inhibited above 10^{-5} M. There is little or no aerobic fermentation with low concentrations of inhibitor, its appearance being associated with concentrations that inhibit respiration. Over a narrow concentration range between about 2 and 3×10^{-5} M. the rate of aerobic fermentation jumps from 50 to 126 per cent., while there is a decrease in the respiration rate from 154 per cent., almost the peak value, down to 120 per cent. Further increases in concentration result in an inhibition of aerobic fermentation as well as respiration.

From the figures of oxygen uptake in respiration and carbon dioxide output in aerobic fermentation can be calculated the rates of glucose catabolism assuming that the two processes obey the usual equations at all inhibitor concentrations—

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

The values so obtained are plotted in Fig. 1 A as percentages of the rate of glucose breakdown in the control. A curve with a single and very marked peak results. The rate of carbohydrate breakdown increases rapidly up to a concentration of 3×10^{-5} M., a concentration at which the rate of respiration is already falling.

A comparable set of experiments was carried out over a limited range of dinitrocresol concentrations by Krahl and Clowes (1935), who measured oxygen uptake and alcohol production. Their figures have been plotted out in Fig. 2 A together with the calculated rate of glucose catabolism. These experiments were made at pH 4·45, a pH only slightly less than that used in the present experiments, and it will be seen that there is a close similarity in the curves obtained. Particularly striking is the rise in alcohol production associated with inhibition of respiration and corresponding to the rise in carbon dioxide of aerobic fermentation found in the present experiments.

The effect of dinitrocresol on anaerobic fermentation, i.e. carbon dioxide output under nitrogen, was next studied. The rate of anaerobic fermentation is shown in Fig. 1 B as a percentage of the rate of carbon dioxide production by controls in air. In the controls the rate of fermentation is just under twice the rate of respiration: at about 10^{-5} M. dinitrocresol there is a small degree of stimulation and higher concentrations inhibit carbon dioxide production. From these results the corresponding rates of carbohydrate destruction under nitrogen are readily calculated; the control rate is nearly 6 times that in air, and the curve follows the same trend as that for the rate of fermentation.

A corresponding set of measurements was made on the effect of cyanide on respiration (Fig. 2 B). With increasing concentrations the rate of respiration is progressively reduced, although no clear evidence was obtained for a cyanide-stable respiration (Commoner, 1940). As respiration is inhibited so aerobic fermentation appears, its magnitude increasing slowly at first, but more rapidly as respiration is finally quenched. From these two curves can be calculated the rate of glucose destruction. At first it follows the reduction in respiration rate, reaching a minimum value of 82 per cent. of the control

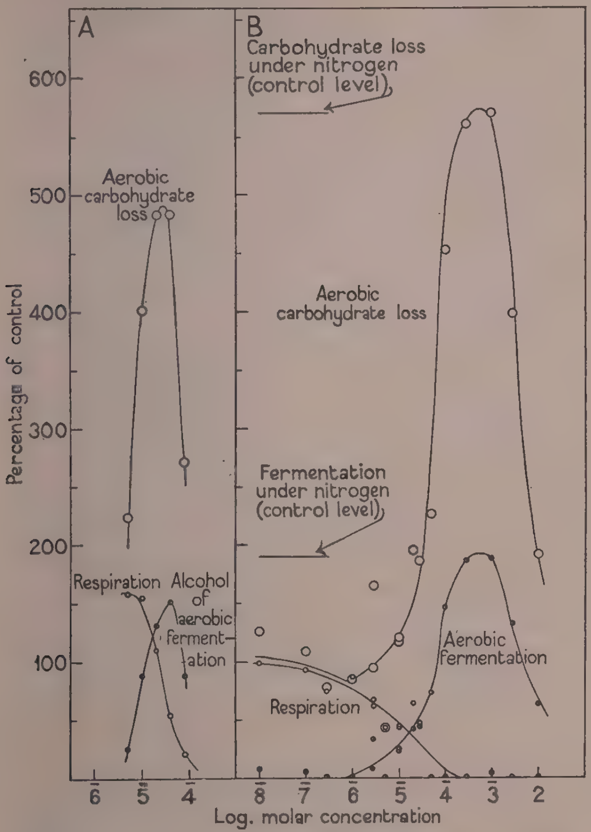

FIG. 2. A. Effect of 3:5-dinitro-*o*-cresol on oxygen uptake, alcohol production, and total carbohydrate consumption by yeast at pH 4·45. Data of Krahl and Clowes, 1935. Alcohol has been treated as a gas (1 Mol = 22·4 l.) and plotted as percentage of oxygen uptake in the controls. B. Effect of hydrogen cyanide at pH 5·0 in air on oxygen uptake, aerobic fermentation, and carbohydrate destruction by yeast. Expressed as in Fig. 1 A.

rate at 8×10^{-7} M.; further increases in concentration cause a rapid increase in glucose breakdown as aerobic fermentation increases in magnitude. Finally, above 10^{-3} M. there is an inhibition of the rate of fermentation and so of the rate of loss of carbohydrate.

DISCUSSION

Relations between respiration and the appearance of aerobic fermentation. In the experiments with cyanide the appearance of aerobic fermentation is clearly associated with inhibition of respiration. The same result was obtained by Marsh and Goddard (1939), who measured the effect of a wide range of cyanide concentrations on the respiration and aerobic fermentation of carrot tissue. The experiments of Genevois (1927), who claimed that cyanide would release aerobic fermentation without inhibiting respiration in *Lathyrus*, have recently received just criticism from Turner (1951).

With increasing concentrations of dinitrocresol, on the other hand, the respiration of yeast is first increased and then suffers a reduction. It has been argued previously (Simon, 1953*a*) that two separate processes are to be distinguished—one of stimulation and one of inhibition. On this basis, it is again the case that the appearance of aerobic fermentation is associated with the phase of respiratory inhibition. Earlier experiments on glucose assimilation by yeast gave a comparable result (Simon, loc. cit., Fig. 9): a resting suspension of yeast will only ferment an appreciable fraction of the glucose supplied to it in air if the concentration of dinitrocresol is sufficient for a quite marked inhibition of respiration.

At dinitrocresol concentrations below those evoking the peak of respiration there is only a very small amount of aerobic fermentation in the present experiments. However, Beevers (1953) found that aerobic fermentation appears at dinitrophenol concentrations less than are required for maximum stimulation of the respiration of corn coleoptiles. This was attributed to a stimulation of the rate of carbohydrate breakdown or glycolysis, resulting in the production of pyruvic acid at a rate which saturated the pathway of oxidation even in the absence of inhibition; some pyruvic acid was consequently forced to enter the pathway of fermentation.

It may be concluded from these experiments that aerobic fermentation only appears when the pathway of respiration is saturated; with cyanide this occurs as a result of oxidase inhibition, while with dinitrocresol saturation may be due to inhibition of oxidation enzymes or to the increased rate of glycolysis.

Much work has been done on the mechanism of the inhibition of respiration by cyanide. The reactions of cyanide with cytochrome oxidase have been studied by Stannard and Horecker (1948) and with ferricytochrome *c* by George and Tsou (1952). The inhibitory action of dinitrophenols is less well understood, although there is some evidence that they act on flavoprotein enzymes (Krahl *et al.*, 1940; Haas *et al.*, 1942).

Inhibition of aerobic and anaerobic fermentation. Measurements of both aerobic and anaerobic fermentation were undertaken in the experiments with dinitrocresol, and it is clear from Fig. 1 B that the two processes are inhibited by the same concentrations, as is indeed to be expected on the premiss that they are biochemically identical. The same was found in experiments with dried yeast preparations by Meyerhof and Fiala (1950). The mechanism of inhibition is not yet entirely clear. Vandendriessche (1941–2) found that carboxylase is inhibited by several dinitrophenols, but was unable to exclude the possibility that other enzymes are also affected.

Although no experiments were made with cyanide under nitrogen, some indication that aerobic and anaerobic fermentation in yeast are equally sensitive to cyanide comes from the observation of Warburg (1949) that the rate of anaerobic fermentation is halved by 10^{-2} M. cyanide, which is only a little more than is needed for an equivalent inhibition of aerobic fermentation (Fig. 2 B). Meyerhof and Kaplan (1952) attribute the action of cyanide on yeast fermentation mainly to an inhibition of the metal-free enzyme alcohol dehydrogenase, but it may also interfere at several points in the glycolytic cycle by its action as a carbonyl reagent.

Rate of carbohydrate destruction. The rate of carbohydrate loss in air and in nitrogen was calculated from the rates of oxygen uptake and carbon dioxide output. With cyanide, the rate first falls as respiration is inhibited and then rises with the appearance of aerobic fermentation.

With dinitrocresol there is no preliminary fall in the rate of carbohydrate loss. The rate increases rapidly until the concentration is reached at which it becomes limited and then reduced as the pathway of fermentation is inhibited. Since the rate of carbohydrate loss reaches its peak at a concentration (3×10^{-5} M.) higher than that required for the maximal respiration rate (10^{-5} M.), it follows that over this range, while the pathway of metabolism switches from the oxidative to the fermentative, the rate of glycolysis (conversion of carbohydrate to pyruvic acid) is yet increasing.

Significance of the stimulation of glycolysis with dinitrocresol. The chief difference between the results with dinitrocresol and those with cyanide lies in the stimulation given to respiration by dinitrocresol but absent with cyanide. A preliminary analysis of this action of dinitrocresol was made in the previous paper (Simon, 1953*a*), where it was shown that stimulation is accompanied by an inhibition of the assimilation of glucose, a process for which energy is required. As the concentration of dinitrocresol was raised, the stimulation of respiration and the inhibition of assimilation both became more pronounced. This relationship only held up to the concentrations at which respiration became inhibited. With the present results the argument can be carried farther by comparing the effect of dinitrocresol on glycolysis with its effect on glucose assimilation. The curve obtained earlier for the inhibition of the oxidative assimilation of glucose by yeast at pH 5·0 is plotted out in Fig. 1 B. While it is clear that the progressive inhibition of glucose assimilation is reflected in the stimulation of glycolysis, up to the concentration at which glycolysis becomes inhibited, it is difficult to establish from this graph whether increasing concentrations of dinitrocresol affect the two processes equally or not. Comparison of the two curves would be easier if one of them were inverted. In Fig. 3 A the curve for glycolysis has been inverted by plotting the rate of glycolysis in the controls as a percentage of that in the presence of dinitrocresol, and it will be seen that stimulation of glycolysis is exactly paralleled by inhibition of assimilation. A given concentration of dinitrocresol increases glycolysis rate by as much as it inhibits assimilation. It may therefore be presumed that these two actions of dinitrocresol have a common cause.

The nature of this effect of dinitrocresol on metabolism has been elucidated by research with isolated enzyme systems (Simon, 1953*b*). Briefly, it has been found that dinitrocresol has the power of preventing the formation of energy-rich phosphate during oxidation, or 'uncoupling' phosphorylation. As a result less energy-rich phosphate is available and endergonic processes are inhibited. Under normal conditions the rate of glycolysis is believed to be controlled by the availability of acceptors for energy-rich phosphate, increasing as energy is utilized in synthetic processes. In the presence of dinitrocresol respiration becomes a less efficient phosphorylating mechanism and glycolysis can proceed faster without saturating the supply of phosphate acceptors. A given concentration of dinitrocresol reduces the rate of phosphorylation to a particular level, decreasing endergonic processes such as assimilation and increasing the rate of glycolysis by corresponding amounts.

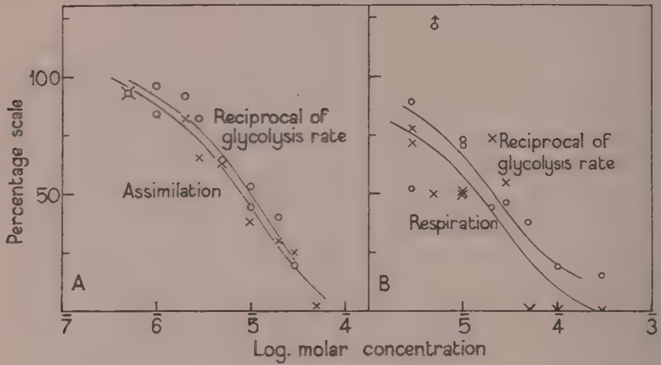

FIG. 3. A. The effect of 3:5-dinitro-*o*-cresol on the rate of glycolysis of yeast in air compared to its effect on the oxidative assimilation of glucose. Assimilation is expressed as a percentage of the control: glycolysis rate is plotted as a reciprocal, the control rate being given as a percentage of the rate in the presence of dinitrocresol. B. The effect of hydrogen cyanide on the rate of respiration of yeast and on the reciprocal of the glycolysis rate.

The present experiments thus confirm the view that the phosphate cycle acts as a regulator of glycolysis rate not only in the various cell-free systems in which it has been most investigated but also in the living yeast cell.

We may now consider the relationship between the rates of glycolysis in air and in nitrogen. In the controls the rate of glycolysis in air is about one-sixth of that in nitrogen. Increasing concentrations of dinitrocresol stimulate the rate in air until at 5×10^{-5} M. and above it is equal to that in nitrogen or even a little greater. The rise in the rate of aerobic glycolysis is paralleled by the increased inhibition of assimilation, from which we may infer an increased degree of uncoupling; at 5×10^{-5} M. assimilation is strongly inhibited and uncoupling therefore almost complete. Hence it appears that by reducing the efficiency of respiration as a phosphorylating mechanism the fast anaerobic rate is reached. The rates of aerobic and anaerobic glycolysis at 5×10^{-5} M. dinitrocresol are 5 times as fast as the air control, but it could well be argued that were it not for the inhibition of fermentation at this high concentration the rate of glycolysis in air would rise fully 6 times to equal the rate in controls under nitrogen. From this point it is but a small step to the supposition that carbohydrate breakdown in baker's yeast is fast under nitrogen because it is then relatively inefficient as a phosphorylating mechanism, only two energy-rich phosphates being formed per mole of glucose degraded in place of the larger number formed per mole in air.

The results of the dinitrocresol experiments therefore support the view, well expressed by Johnson (1941), that aerobic glycolysis is slower than anaerobic because it is more efficient as a phosphorylating mechanism.

The results of the cyanide experiments can also be interpreted in terms of the phosphate cycle. With low concentrations of cyanide the rate of glycolysis is reduced as respiration rate falls. With concentrations of 10^{-6} M. and above respiration is inhibited more strongly and the level of energy-rich phosphate becomes reduced to a point at which glycolysis can proceed more rapidly. A comparison of the effects of these concentrations of cyanide on respiration and on glycolysis rate (Fig. 3 B) indicates that it affects the two processes in nearly equal measure. Yeast fermentation is relatively insensitive to cyanide and the rate of glycolysis in air increases up to fully 575 per cent. of the air control with 5×10^{-4} M. cyanide.

A powerful analogy can now be drawn between inhibition of respiration by cyanide and inhibition of glucose assimilation by dinitrocresol since both are measures of the level of energy-rich phosphate and both bear a direct relation to the rate of glycolysis. Only the first part of the peaked curve of respiration obtained with dinitrocresol is related to glycolysis rate and the production of energy-rich phosphate. Cyanide lowers the rate of production of energy-rich phosphate by slowing the rate of respiration; dinitrocresol dislocates the mechanism of production of energy-rich phosphate while allowing the respiration to proceed more rapidly.

The experimental results can thus be interpreted in terms of the phosphate cycle as the mechanism which regulates glycolysis rate. However, the results of the cyanide experiments could be interpreted equally well along the lines put forward by Gottschalk (1941). It is then supposed that the reduced DPN produced during respiration is only oxidized slowly by atmospheric oxygen and that the relatively low level of oxidized DPN is the factor which limits aerobic glycolysis. When the terminal oxidase system is inhibited with cyanide, reduced DPN becomes oxidized by converting acetaldehyde to alcohol, a process which is supposed to be more rapid, so that the proportion of oxidized DPN rises and the rate of glycolysis can increase. It appears difficult, however, to interpret the dinitrocresol results in the same way. If the rate of glycolysis in air is limited by the concentration of reduced DPN there seems no obvious reason why the rate should increase in the presence of dinitrocresol since this compound is not known to have any stimulating effect on the terminal oxidation processes as such.

The evidence therefore points to the phosphate cycle as the factor controlling glycolysis rate in yeast. However, it is to be noted that the actions of cyanide and dinitrocresol on yeast respiration and fermentation are in no wise exceptional, as comparable observations have been made with the tissues of higher plants (Marsh and Goddard, 1939; Beevers, 1953; also Simon, 1953*b*), and one is therefore led to inquire whether the same mechanism could not be responsible for the regulation of glycolysis in yeast and in higher plants.

This work was carried out during tenure of a Senior Studentship awarded by the Royal Commission for the Exhibition of 1851. It is a pleasure to thank Professor T. A. Bennet-Clark, F.R.S., for stimulating discussion.

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Effect of Temperature and Desiccation during Storage on Germination and Keeping Quality of *Kochia indica* Seeds

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SUMMARY

Data are given for *Kochia indica* seeds showing retention of viability after storage for various periods of time open to the air under laboratory conditions, open at 30° C., open at 38° C., and sealed over $CaCl_2$ at 30° C.

Seeds have been stored without deterioration at 30° C. sealed over $CaCl_2$ for over 14 months.

Rapid deterioration of seed in open storage at laboratory temperature and at 30° C. shows that loss of viability is accelerated by moisture more than by temperature.

INTRODUCTION

PRELIMINARY tests in 1951 revealed that seeds of *Kochia indica* collected the previous autumn and kept in open storage under laboratory conditions showed signs of abnormality by May, and by August had lost their vitality completely. This is in itself of interest in relation to the maintenance of the species in the Egyptian climate. The effect of various storage conditions on the viability of these seeds appeared therefore to be worth investigating as not only of academic interest but perhaps of some economic importance in relation to its possible use as a fodder plant (El-Shishiny and Thoday, 1953).

No attempt will be made here to cite all the published work on longevity of seeds, but a few papers may be referred to which appear relevant to the present investigation. A full review of the subject has been given by Crocker (1938, 1948).

Certain seeds are known to lose their vitality soon after harvest when left in open air. Until recently this rapid loss of vitality was supposed to be due mainly or solely to the drying effect of the air. Data now available on many different seeds demonstrate conclusively that the longevity of many seeds in storage is increased by drying, although others do not tolerate desiccation.

Death by ageing in some of these may be the result of partial desiccation of the protoplasm of the embryo if they are kept in open air after harvest. The loss of viability by ageing in seeds which remain viable when thoroughly dry cannot be the result of consumption of reserve food material, since most seeds which have lost viability during periods of prolonged storage are found still to contain an abundant food-supply. The death in this case is more

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probably associated with the accumulation of certain more or less toxic products of respiration (Crocker, 1948). Meanwhile there are enough data which demonstrate that complete removal of water is injurious to most seeds and that the degree of drying endured without injury varies with different seeds.

The work of Jones (1920) on *Acer*, Duvel (1905) on *Zizania*, Joseph (1929 *a* and *b*) on *Betula*, Barton (1943) on *Citrus* give examples of loss of viability due to desiccation to a range of low water contents. On the other hand, Nakajima (1925) on *Salix*, Kearns and Toole (1938) on *Festuca*, Darragh (1931) on sugar-cane all give examples of increased viability as a result of desiccation.

MATERIAL AND METHODS

As soon as the new season's crop of seeds became available at the end of October a collection was made, continuing through November and December 1951, and systematic experiments were begun. Those dealing with the conditions of germination have already been described (El-Shishiny and Thoday, 1953). This paper is concerned with the duration of viability and the conditions that affect it.

As the temperature in Alexandria even in spring may rise at times considerably and in summer may reach 30–35° C., and the relative humidity may reach as high as 75 per cent., samples of seed stocks were stored under the following conditions: (1) in the laboratory open to air; (2) at 30° C. in an oven open to air; (3) at 30° C. in an oven in a wide-mouth glass-stoppered bottle over $CaCl_2$; (4) at 38° C. in an oven open to air.

This was planned to see whether the rapid deterioration of seeds stored in the open is the result of high temperature or high relative humidity during the summer.

The seeds of *Kochia indica* are not dormant but begin to germinate as soon as they are submerged in water a few millimetres deep. The seeds kept under the different storage conditions were tested for germination at intervals by submerging samples, usually of 15 seeds, in shallow water in Petri dishes for 24 hours—at 20° C. in the winter months and at laboratory temperature in the summer.

In addition to the regular germination tests, it seemed of interest to determine whether old seeds will produce normal seedlings. For this purpose the rate of elongation of seedlings was determined by measuring the length of seedlings from the root-tips to the cotyledonary node at 24-hourly intervals from the beginning of the germination test.

RESULTS AND DISCUSSION

Percentage germinations of seeds after storage open at laboratory temperature, 30° C., 38° C., and sealed over $CaCl_2$ are shown graphically in Fig. 1 for different periods of storage from 31 October 1951.

Table I gives data of the lengths of seedlings produced from seed stored under the different conditions studied. It will be noted that growth rates are much reduced in the case of seeds stored in the open in the laboratory.

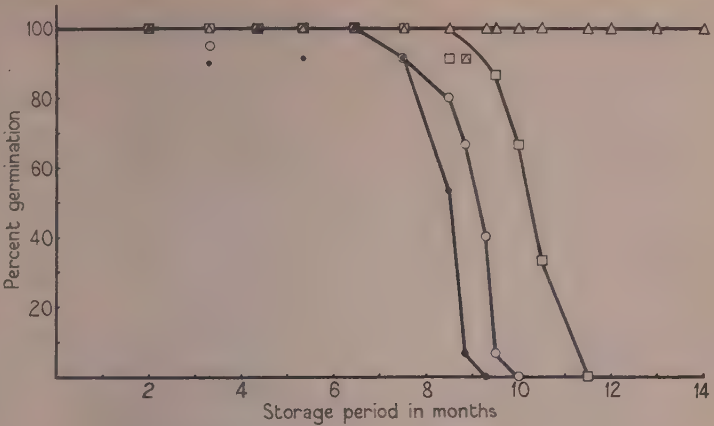

FIG. 1. Effect of storage conditions on viability of *Kochia indica.*
—●— open storage; —○— open 30° C.; —□— open 38° C.; —△— $CaCl_2$ 30° C.

TABLE I

Length of seedlings of Kochia after 1, 2, and 4 days, percentage germination shown in parentheses. Time of storage, 256 days

	Days' germination		
Storage conditions	1	2	4
Open lab. temp.	2·4 (33)	3·0 (40)	3·8 (53)
Open 30° C.	3·0 (67)	4·0 (80)	5·0 (80)
Open 38° C.	3·4 (93)	4·3 (93)	6·4 (93)
$CaCl_2$ 30° C.	8·3 (100)	21·5 (100)	30·0 (100)

Open storage at 30° C. is superior to laboratory open storage. Here seeds kept their viability unimpaired for 7½ months like the seeds at laboratory temperature, but after that the fall in viability was less steep. Open storage at 38° C. is far superior to open storage either at laboratory temperature or at 30° C.

Certainly the moisture content of seeds air-dried at 38° C. is lower than at 30° C. or laboratory temperature. The rapid deterioration of seeds under laboratory open storage conditions is most probably due to high moisture content of the seeds or to moisture fluctuation, as wide variations in water content may take place in open storage. Barton (1943) has shown that not only is the high moisture content injurious for keeping quality of *Citrus* seeds but fluctuations in moisture content are also detrimental. Steinbauer (1937), as a result of his experiment with *Fraxinus*, concluded that these seeds are much more sensitive to moisture content than to temperature.

Definite reduction in the capacity for growth was evident after 7½ months' storage at laboratory temperature, 30° C., and 38° C. The seeds have low vigour though still viable. They may be able to germinate under favourable conditions, but the seedlings make little or no further growth. This result is in general agreement with those of Parkinson (1948) and Barton (1932, 1939 *a* and *b*, 1946). Under unfavourable conditions of storage, the embryo of the seed may become weakened and produce inferior or abnormal seedlings. In fact, many of the seedlings of *Kochia indica* died before the radicle was more than a few millimetres in length. The growth, moreover, was abnormal; instead of being straight, the radicles were curved or coiled into spirals.

In contrast with open storage, sealed storage over calcium chloride has kept the viability of *Kochia indica* seeds quite unimpaired for 15 months, and germination tests are still being continued. The seedlings produced are, moreover, healthy and vigorous as shown by growth-rate measurements. Thus *Kochia indica* is to be classed along with sugar-cane and *Salix* spp., the seeds of which also retain their vitality in a dry atmosphere.

The following data of the water content as percentage dry weight of seeds of *Kochia indica* stored at different temperatures and over $CaCl_2$ at 30° C. are relevant to the results here reported. Open storage, at lab. temp., 10·5 per cent.; at 30° C., 4·8 per cent.; at 40° C., 3·1 per cent.; and over $CaCl_2$, at 30° C., 0·87 per cent.

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